Assessment of cancer risk using human fibroblast cells(Dissertation_全文)

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Preface

The study presented in this thesis has been carried out under the direction of Professor Saburo Mastui at the Laboratory for Control of Environmental Micropollutant, Kyoto University, and Professor Hiraku Takebe at the Department of Radiation Genetics, Kyoto University during 1992-1994. Throughout the course of the research herein described the author has had the continual good fortune to be in contact with people who provided useful suggestions, encouragement and aid of many kinds. The author would like to express his gratitude to all of them for their many contributions to the success of this work.

The auther wishes to express his sincerest gratitude to Professor Saburo Mastui and Professor Hiraku Takebe for their constant guidance and encouragement throughout this work. The author wishes to express his deep gratitude to Dr. Takashi Yagi, Associated Professor of Dep. of Radiation Genetics of Kyoto Univ., for his continuing advice and stimulating discussion during the course of this study. The author deeply thanks to Associated Professor Kounosuke Nishida and Assistant Harumi Yamada, Lab. for Control of Micropollutant Kyoto Univ., for their valuable suggestion. The author would like to thank Mr. Masanobu Kawanishi for his active collaborations.

Last but not least, the author wishes to express his deep appreciation to his parents, Mr. Yasuhei Matsuda and Mrs. Yukiko Matsuda, for their constant assistance and encouragement.

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Contents

Chapter 1

Strategies for Risk Assessment of Environmental Carcinogenic Factors to Human Beings

1. The First strategy: Genotoxicity tests using human cells

To know the impact of the environmental carcinogenic factors on human cancers, the first strategy we propose in this thesis is the development of bioassay using human cells.

Many genotoxicity tests were developed to estimate the risk of human cancer. These tests are classified roughly into bacterial assay, in vitro assay using animal cells, in vivo assay using experimental animals, in vitro assay using cultured human cells and biomonitoring of human beings or epidemiological studies. Carcinogenicity studies in experimental animals and epidemiological studies provide us the most reliable data for the risk assessment of human cancers. There are some researchers who claim that most of the studies, except these two studies, are not useful for assessing the cancer risk. Taking account of the numerous costs and the time consumption, however, many other researchers begin to consider that the cancer risk should be estimated by the data of short-term genotoxicity tests. Idealy, the sensitive assay which can detect mutations occuring in tumor-related gene is best for this purpose, however, which has not been

developed so far. The established tests available now are listed in table 1. The periods of time required to carry out these tests are roughly 1 year for the carcinogenicity tests, 1 month for the gene mutation assays, 1 week for the clastogenicity assays, and 2-3 days for DNA damage assays.

Mechanisms of DNA strand breaks

It has been shown that DNA strand breaks are induced by following three mechanisms.

First, strand breaks occur directly by physical (ionizing radiation) and chemical (reactive oxygen, bleomycin etc.) agents. The majority of these breaks are caused by breakage of phosphodiester linkages in one of the polynucleotide chains, and destruction of the deoxyribose ring can also result in interruptions of the deoxyribose-phosphate backbone. The precise molecular mechanisms of DNA strand breakage induced by ionizing radiation are various and complicated. They may involve direct ionization of the phosphodiester bond, or fragmentation reactions involving the bases or sugars, coupled with electron rearrangements that ultimately result in hydrolysis of labilized phosphodiester bonds(1).

Second, strand breaks also occur by repair endonucleases. For example, ultraviolet light irradiation do not induce strand breakage directly, but produce crucial DNA damage "pyrimidine dimer". Living organisms have

Table 1 Established studies for evaluation of human cancer risk

Classes of studies	Animal		Human	
	in vitro	in vivo	in vitro	in vivo
DNA damage				
DNA strand break	⊙	○	⊙	△
unscheduled DNA synthesis (UDS)	⊙	○	⊙	△
DNA adducts (^{32}P-postlabel)	⊙	⊙	⊙	◔
Clastogenicity				
chromosomal aberration	○	○	○	△
sister chromatid exchange (SCE)	○	○	○	△
micronucleus assay	×	⊙	×	△
Gene Mutation				
APRT	○	○	×	×
using transgenic animals	×	⊙	×	×
HPRT	×	×	○	○
Carcinogenicity				
focus formation assay	○	×	△	×
carcinogenicity study	×	⊙	×	×
epidemiological study	×	×	×	○

⊙ ; very useful, ○ ; useful, △ ; not useful because of low sensitivity × ; can not apply

enzymatic systems for removing this damage, i.e., DNA repair systems. The most general DNA repair system observed in nature is the DNAexcision repair which excises damaged or inappropriate nucleotides from DNA and replaces with the normal nucleotides. The excision repair endonucleases make an incision in DNA by catalyzing the hydrolysis of phosphodiester bonds at the site of damaged base(2).

Third, DNA strand breaks are induced at the apoptotic cell death. The apoptosis is induced by developmental control signals, and can also be induced by toxic insults, particularly by DNA damage. In apoptotic cells, DNA fragmentation is occured by Ca^{2+} or Ca^{2+}/Mg^{2+}-dependent endonuclease(3,4).

As mentioned above, DNA strand break may be a useful analytical parameter for assessing the exposure of the genetic material to genotoxic chemicals.

2. The Second Strategy: Molecular Epidemiological Approach

The crucial differences between normal cells and cancer cells at the molecular level stem from discrete changes in specific genes controlling proliferation and tissue homeostasis. Over 100 such cancer-related genes have been discovered, several of which are implicated in the natural history of human cancer because they are consistently found to be mutated in tumors. Analysis of mutations in tumors is an exceptionally useful tool in the epidemiology of human cancer. Although it is now clear that changes in specific DNA sequences in the cancer-related genes lead to cancer, the agents that induce these changes in humans are still much debated. Sequence changes in genes can be generated by both exposure to DNA damaging agents such as electrophilic mutagens, and spontaneously from intracelluar biochemical and enzymatic processes. Each mutagen or mutagenic process produces mutagen-specific alterations as finger prints in DNA, with respect to the nature of the changes, the locations of the changes, and the frequencies of the alterations in the gene. An analysis of the spectrum of mutation is providing an empirical approach to the fundamental question: What causes genetic changes in cells? Data compiled on mutations have already revealed several examples of human tumors consistent with the fingerprints of DNA damage induced experimentally by known carcinogenic risk factors. For example, ultraviolet radiation leaves

the most distinctive fingerprint in DNA: unrepaired cytosine dimers induce tandem mutations, in which two adjacent cytosine residues are replaced by two thymine bases. These mutations occurs very rarely unless cells are exposed to ultraviolet radiation(5,6). Some of the mutations discovered in the p53 tumor suppressor gene of squamous-cell carcinomas in the skin in sun-exposed area were in agreement with such tandem substitutions(7,8), directly incriminating exposure to ultraviolet light.

Use of *supF*, the *E.coli* Tyrosine Suppressor tRNA Gene, as a Mutagenic Target in Shuttle-Vector Plasmids

To know the mutation spectrum of the carcinogens, the *Escherichia coli* tyrosine amber suppressor transfer RNA gene, *supF* has been used as a mutagenic target in the shuttle vector plasmids such as pZ189 and pS189(5,9-26). The anticodon in *E. coli* tyrosine tRNA is GUA. In *supF* tRNA,a G to C transversion mutation changes the anticodon to CUA which inserts tyrosine into the (amber) chain termination (nonsense) codon, UAG(27,28). *supF* thus functions as a suppressor tRNA enabling to read through of the UAG stop codon.

The supF gene consists of the promoter region (base pairs 24-58), a precursor RNA region involved in RNA processing (base pairs 59-98), the suppressor tRNA sequence (base pairs 99-183) and a 3' flanking region (base

pairs 184-200) (Fig.1). The base pair number of the supF gene is that from the unique EcoRI site in pZ189 in this thesis.

Bacterial strains possessing amber mutations can be used as indicator strains for measuring the function of the plasmids containing *supF* gene.

Escherichia coli K12 strain KS40 and plasmid pKY241 were designed for easily screening the supF mutations contained in the shuttle vectors(29). KS40 is a nalidixic acid-resistant (*gyrA*) derivative of MBM7070 (*lacZ* (am) *CA7070 lacY1 HsdR HsdM Δ(araABC-leu)7679 galU galK rpsL thi*), which has been used for detection of the mutated pZ189(30). Plasmid pKY241 was constructed by Dr.Akasaka et al. and contains a chloramphenicol resistant marker and a *gyrA*(amber) gene. When KS40 containing pKY241 (designated KS40/pKY241) is transformed with *supF* gene, nalidixic acid-resistant GyrA protein is produced both from the chromosomal *gyrA* gene and wild type GyrA protein from pKY241 because of the suppression of *gyrA* amber mutation by *supF*. It is known that the nalidixic acid-sensitive phenotype (wild type) is dominant over nalidixic acid-resistant phenotype(mutant type). Thus, KS40/pKY241 gives rise to nalidixic acid-sensitive colonies when it carries a active *supF* gene. *E.coli* KS40/pKY241 cells carrying active *supF* suppressor tRNA are sensitive to nalidixic acid, whereas the cells carrying mutated *supF* form colonies on plates containing nalidixic acid, chloramphenicol and ampicillin. IPTG and X-gal were further

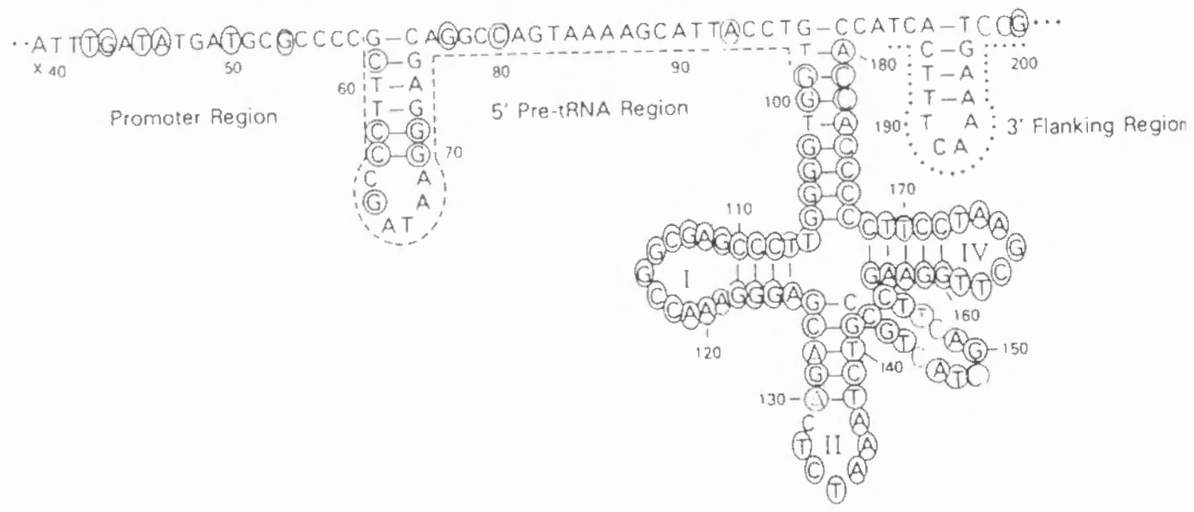

Fig. 1 Hypothetical secondary structure of a single strand DNA containing the *supF* tRNA gene sequence (modified from ref. 9). Sites of single base substitution are indicated (circle).

added to confirm selection of the mutated *supF* gene. *E.coli* cells containing active *supF* gene produce blue colonies, whereas the cells having mutated *supF* gene produce white or light blue colonies, because *E.coli* KS40 has an amber mutation in the β-galactosidase gene lacZ.

The possible targets of the supF gene

The mutation spectrums in *supF* gene induced by many carcinogens have been explored by elegant earlier studies. Several classes of base substitution mutants have been described in the supF gene. Base substitution mutations that inactivate suppressor function have been found to include single base substitutions, tandem base substitutions, and multiple base substitutions. Table 2 lists the sites of single base substitution mutations that can inactivate supF function (5,9-26). The supF gene is a very sensitive target for mutagenic inactivation. All 6 possible base-substitution mutations may be monitored and most sites can detect all possible 3 base-substitutions. The extreme sensitivity of the supF gene to mutagenic inactivation, in conjunction with the sensitive KS40/pKY241 indicator assay, makes the *supF* gene a suitable taget for mutagenesis studies with shuttle-vector plasmids.

Table 2 Single base substitutions that inactivate supF function

Position	Type of mutation	Reference
42	T to C	9
43	G to A	9,11,19,26
43	G to C	9
45	T to A	9
45	T to C	9
46	A to T	9
46	A to G	9
50	T to A	9
50	T to G	9
50	T to C	9
53	G to A	16
59	C to A	26
62	C to T	26
63	C to A	9
65	G to A	9,12,14
65	G to T	9
65	G to C	12
70	G to A	Chapter 3 of this thesis
71	G to A	14
71	G to C	9
77	G to A	9,19
80	C to T	12
93	A to T	12,14
99	G to T	12,14,21
100	G to A	9
102	G to A	13
102	G to T	13
102	G to C	10
103	G to A	24
103	G to T	9, 13
103	G to C	10
104	G to A	24
104	G to T	5
104	G to C	9,10
105	G to A	9,24
105	G to T	9,10
105	G to C	5,25
106	T to A	9,21
106	T to G	9,21,25
106	T to C	9,21
107	T to A	24
108	C to A	25
108	C to T	9,11,14,19,23,24
108	C to G	9,25,26
109	C to A	9,10,13,14,17,21
109	C to T	9,19,24,
109	C to G	9,10,12,14,21,24
110	C to A	9,13,18,19
110	C to T	9
110	C to G	19,23
111	G to A	9,11,24
111	G to T	18
111	G to C	14
112	A to T	9,10,17,18,21
112	A to G	9,17,21,24,25
112	A to C	5,9,21
113	G to A	9,11,13,24
113	G to T	10,14,21,25
113	G to C	9,10,13,19,21
114	C to G	9,14
115	G to A	9, 24
115	G to T	10
115	G to C	9, 13,17,25
116	G to A	14
116	G to T	9,10,13,
116	G to C	17,19

Position	Type of mutation	Reference
117	C to A	9,23
118	C to A	9,21
118	C to T	12
118	C to G	9
119	A to G	9
119	A to C	9,21
120	A to T	5,9,12,19,21
120	A to G	5,9,19,21,22,24,25
120	A to C	9,19
121	A to T	15
121	A to G	24
122	G to A	9,19,24
122	G to T	9,13,19,21,23,24
123	G to A	5,9-12,14,20,21,25,26
123	G to T	9,10,12-14,17,20,21
123	G to C	9,10,12,14,21,24,25
124	G to A	5,9
124	G to T	26
124	G to C	9,10,12,21,23,25
125	A to T	9
125	A to G	24
126	G to A	9,24
127	C to A	9,10,13,17,21,25
127	C to T	24
127	C to G	5,9,13,17,21
128	A to T	18
129	G to A	9,20,22
129	G to T	9,17
129	G to C	9,10,12-14,17,19,21,22
130	A to G	24
132	T to G	9,24
132	T to C	9,21,24
133	C to A	5,9,12,13,15,17,19,21,25
133	C to T	9,11,19
133	C to G	5,9,10,12-15,17,19-21,25
134	T to A	9,15,21,25,26
134	T to G	15,21
134	T to C	5,9,19,21,24,25
135	A to T	5,9,15,21,25,26
135	A to G	5,9,21,22,26
135	A to C	9,15,19,21,26
136	A to T	5,9,10,15,17,19,21,22,26
136	A to G	24
136	A to C	9,12,14,19,21,23,
137	A to T	5,9,19
137	A to G	9,10,21
138	T to C	24
139	C to A	9,10,13,17,21,22
139	C to T	9,19,24
139	C to G	5,9,10,12,14,17,19,21,25
140	T to A	9,12,21
140	T to G	9,13,21
140	T to C	18
141	G to A	15,24
141	G to T	9
141	G to C	5,9,13,21,25
143	C to A	9,13
143	C to G	9
144	G to A	9,24
144	G to T	10,17,21,24
144	G to C	5,9,10,12,14,17,21
145	T to C	24
147	A to T	22
148	T to C	Akasaka et al. in press
149	C to A	Akasaka et al. in press
149	C to T	14

Position	Type of mutation	Reference
149	C to G	5,22
150	G to A	9,19,24
150	G to C	9
151	A to G	24
153	T to C	24,26
154	T to A	9,19,22
154	T to G	9
154	T to C	24
155	C to A	5,9,10,17,19-22,24
155	C to T	5,9,11,13,14,17,19,21,23,26
155	C to G	21
156	G to A	5,9,11-14,19-26
156	G to T	5,9,10,13,17,19-22,25
156	G to C	5,12,13,21,25
157	A to G	24
158	A to T	9,21
158	A to G	24
158	A to C	9,19,21
159	G to A	5,9-15,17,19-23,25,26
159	G to T	5,9,10,12-14,17,20-22,25,26
159	G to C	9,12,17,19
160	G to A	5,9,11-13,15,17,19,21,25
160	G to T	5,9,10,12-14,17,18,21,25
160	G to C	12,13,21
161	T to A	5,9,15,21
161	T to G	5,21
161	T to C	5,9,21,24
162	T to A	9,13,15,21,23
162	T to G	9,13,21
162	T to C	24
163	C to A	9,19,20
163	C to T	9,19,24
163	C to G	9
164	G to A	23,24,26
164	G to T	5,9,10,12-14,17,19,21,23-26
164	G to C	5,9,12-14,17,19,21,25
165	A to T	9,19,21
165	A to G	5,24
165	A to C	9,13,19,21,23
166	A to T	15
166	A to G	24
167	T to A	24
167	T to G	9
167	T to C	24
168	C to A	5,9,10,12-15,17-21,23-25
168	C to T	5,9-15,17-26
168	C to G	5,9,10,14,17,19,21,23,25
169	C to A	9,10,13,17-19,21,25,26
169	C to T	5,9-12,14,18,19,21-26
169	C to G	9,13,17,19
170	T to A	9
170	T to C	24
171	T to A	9,21,26
171	T to C	24
172	C to A	5,9,10,12,13,17,21,25
172	C to T	5,9,14,21,24
172	C to G	21,25
173	C to A	9,21
173	C to T	26
173	C to G	21
174	C to A	9,10,13
174	C to T	24
175	C to T	5,9,19,24
176	C to A	10
177	A to T	21,22
177	A to G	9,21
177	A to C	10
178	C to A	10,13
179	C to A	9,21
179	C to T	9
180	A to T	9
180	A to G	24
200	G to T	9

3. Survey of this thesis

Chapter 2 concerns the application of the DNA strand breaks to short-term genotoxicity test using human cells. The FADU assay has been used for this purpose.

Chapter 3 deals with genotoxicity of tap waters. The actual condition of genotoxic pollution of tap waters and the antigenotoxic factors of them have been described.

Chapter 4 concerns the mutation spectrum of 2-chloroacetaldehyde, the ultimate carcinogenic form of the vinyl chloride.

Chapter 5 deals with the mutation spectrum of acetaldehyde. The impact on human cancers has been discussed.

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Chapter 2

FADU Assay for Evaluation of DNA Damage Caused by Environmental Micropollutants

ABSTRACT

FADU (Fluorometric Analysis of DNA Unwinding) is one of the most simple and sensitive methods to detect DNA strand breaks. We modified the original FADU protocol in this study, and tried to apply the method as a short-term test to detect DNA-damaging environmental agents, using an SV40-transformed human fibroblast cell line WI38-VA13 . DNA strand breaks induced by physical and chemical carcinogens such as γ-rays, ultraviolet light, MNNG, 4NQO, B(a)P were detected efficiently by using the modified protocol. We also tried to detect DNA strand breaks caused by the effluent of a waste water treatment plant. About 5.6 strand breaks per 10^8 base pairs were induced when cells were exposed for 1 h to water sample which was concentrated 200 fold. We conclude that the FADU assay using VA13 human fibroblast cells is useful for detecting DNA damaging substances in the environmental water.

INTRODUCTION

Many bioassays are used to detect and evaluate genotoxicity of environmental micropollutants. To evaluate the risk to human health, it is important to use human cells for the bioassay. Although gene mutation or chromosomal aberration are useful parameters for risk assessment, skill and time consumption are needed for performing these tests. Since DNA strand breakage is easy to measure, and is induced by a variety of chemical and physical agents, and it may be useful as an analytical parameter for assessing the exposure of the genetic material to genotoxic chemicals (1). FADU (Fluorometric Analysis of DNA Unwinding) is one of the most simple and sensitive methods to detect DNA strand breaks (2). We tried to apply this method as a short-term test to detect DNA-damaging environmental agents.

MATERIALS AND METHODS

PRINCIPLE OF THE FADU ASSAY

When double-stranded DNA is exposed to moderately alkaline solution, hydrogen bonds are broken and the 2 strands unwind. For relatively small DNA molecules, the rate of strand-unwinding is very rapid and reaches completion in less than 30 sec (3). By contrast, the very large DNA molecules present in mammalian cells may require hours of exposure to alkaline solution for complete unwinding (4). It has been observed that the rate of unwinding of large DNA molecules in alkaline solution is increased by prior exposure of cells to ionizing radiation. This means that radiation-induced strand breaks are responsible for the increased rate of unwinding and, conversely, that an increased rate of DNA unwinding can be used as a sensitive measure of strand breaks (4-8).

Earlier methods for detecting DNA unwinding in alkaline solution have required physical separation of single-stranded from double-stranded DNA using a hydroxyapatite column, nuclease digestion and precipitation, or filter binding (4-8). In addition, radiolabeling of cells was required for detection of the small amounts of DNA. In cases where radiolabeling was not feasible or to be avoided, sensitive fluorometric methods were substituted to permit detection and quantitation of DNA after column or filter separation (9-12). Birnboim and Jevcak developed a new method for

monitoring DNA unwinding (2). The principle of the procedure is as follows.

The fluorescent dye, ethidium bromide, binds selectively to double-stranded DNA in the presence of single-stranded DNA when short duplex regions in "single-stranded" DNA molecules are destabilized by alkali. Birnboim and Jevcak develop conditions applicable to crude extracts of mammalian cells under which the dye would show fluorescent enhancement preferentially with double-stranded DNA with little interference by RNA, other cell components, or single-stranded DNA. A cell suspension is divided equally among 3 sets of tubes. The contribution to fluorescence by components other than double-stranded DNA (including free dye) is estimated from a blank sample (B) in which the cell extract is first sonicated lightly and then treated with alkali under conditions which cause complete unwinding of low-molecular-weight double-stranded DNA. A second sample is used for estimating total fluorescence (T), *i.e.*, fluorescence dye to the presence of double-stranded DNA plus contaminants. The difference (T-B) provides an estimate of the amount of double-stranded DNA in the extracts. A third sample (P) is used to estimate the unwinding rate of the DNA. The crude cell extract is exposed to alkaline conditions sufficient to permit partial unwinding of the DNA, the degree of unwinding being related to the size of the DNA. The fluorescence of this sample less the fluorescence of the blank (P-B) provides an estimate of the amount of double-stranded DNA remaining. Percent D is given by the relationship

Percent D= (P-B)/(T-B) $\times$100

Chemicals and Buffer Solutions

Composition of the solutions used was as follows- Solution A: 0.5mM EDTA in PBS; solution B: 9M urea, 10 mM NaOH, 2.5 mM trans-1,2-diaminocyclohexane-N,N,N',N' tetraacetic acid, 0.1% w.v sodium dodecylsulphate; solution C: 0.425 volume solution A in 0.575 volume 0.2N NaOH; solution D: 1M glucose, 14mM mercaptoethanol; solution E: 2μg/ml ethidium bromide in 13.3 mM NaOH.

Rat liver 9,000$\times$g supernatant fraction (S9) was obtained from Oriental Yeast, Tokyo, Japan. Composition of the S9-mix used in this study was as follows, 1 mM HEPES buffer pH 7.2, 1 mM $MgCl_2$, 6.6 mM KCl, 0.8 mM NADP, 1 mM D-Glucose 6-phosphate, 4.4 %(v/v) of S9 (protein content; 22.78 mg/ml S9) in serum free MEM.

Cells.

SV40 transformed human fibroblast cell lines were used . A normal human cell line WI38-VA13 (13) was obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in Dulbecco's modified minimum essential medium (MEM) (Nikken, Kyoto, Japan)

supplemented with 10% fetal bovine serum (Hyclone, Logan, UT).

γ-rays Irradiation

Human cells attached to a bottom of a tissue culture flask (75cm^2, FALCON) were washed once with PBS, trypsinised, suspended in 5 ml of MEM (with 10% serum), and counted by a coultercounter. Cells were collected by centrifugation (500g, 5 min), and resuspended by ice-cold Solution A (final cell concentration was 8×10^5 cells/ml). The cell suspension was divided into polypropyrene vial (2 ml, CORNING) and aliquots of this suspension (1.4 ml) were irradiated with various dose of γ-ray by the SHIMADZU RM61 γ-ray irradiation apparatus (^{137}Cs, 4 Gy/min). After irradiation, the cell suspensions are maintained at 0 ℃ to prevent rejoining of DNA strand breaks, and the FADU assay was done as quickly as possible.

UV Irradiation, Chemical Treatment

Cells (1.1×10^6 / dishes) were plated on culture dishes, and incubated in a CO_2 incubator for one night. Cells were washed with PBS and immediately irradiated by various dose of UV light (254 nm). After irradiation, cells were incubated for 3 h in MEM containing 10% serum, 10 μM arabinofuranosyl cytosine (araC) and 2mM hydroxyurea at 37℃ in a CO_2 incubator.

Test chemicals were dissolved in 5 ml of serum-free MEM and exposed to cells for 1 h in a CO_2 incubator. If the S9-mix activation was required, the chemicals were dissolved in S9-mix instead of the serum-free MEM. At this time, if the test chemical was dissolved in DMSO, final concentration of DMSO should not exceed 1 %. After exposure, each plates were washed with PBS, and cells were incubated for 3 h in MEM containing 10% serum, 10 μM araC and 2mM hydroxyurea at 37℃ in a CO_2 incubator. Cells were washed with PBS, trypsinised, centrifuged and redissolved in 1.5 ml of solution A, and the FADU assay was carried out to estimate DNA strand breaks.

FADU assay

Cells were suspended at approximately 8×10^5 cells/ml in solution A and kept on ice. Aliquots (0.2ml) were placed into 2 sets of three15ml polystyrene tubes each labeled T, B or P, and according to treatment, in duplicate. Solution B (0.2ml) was added to all tubes which were incubated for 10 minutes on ice to ensure cell lysis and chromatin disruption. Solution D (0.4ml) was added with mixing to the tubes labeled T to prevent DNA unwinding. Solution C (0.2ml) was gently dispersed into all the tubes which were kept on ice for 30 minutes to initiate unwinding. The tubes labeled B were sonicated to ensure complete disruption of the DNA before all tubes were transferred to a water bath at 60 ℃ for 60 minutes for DNA

unwinding under alkaline conditions (pH12.8). The tubes labeled P were transferred to a water bath at 28 ℃ for 45 minutes. DNA unwinding reaction in the tubes labeled P and B was arrested by the addition of 0.4 ml of solution D to bring the pH to 11.1. Solution E (1.5ml) was added to all tubes and the fluorescence was measured by a SHIMADZU RF-5000 spectrofluorometer (excitation, 520nm; analyzer, 590nm).

The percentage of double stranded (ds) DNA was determined from the fluorescence of the tubes labeled B (background fluorescence), T (total fluorescence due to ds DNA and contaminants) and P (ds DNA remaining following unwinding in alkali) from the fillowing equation:

% ds DNA = (P-B)/(T-B)×100

Concentration of a Water Sample

We took a effluent of a municipal waste water treatment plant of O-City at 22 February 1993. Water sample was contained in a plastic bottle in the amount of 20 liter. After being brought back to our laboratory, water sample was filtrated by a cellulose filter (5A, ADVANTEC) and adjusted to pH 2 with H_2SO_4 and was passed through the Sep-Pak C_{18} cartridge at room temperature. The volume of water passed through per one cartridge was 5 liter and the filtration rate was 4 mL/min. When adsorption was completed, 2 mL of pure water was passed through the cartridge to wash the

resins and remaining water was purged away from the cartridge by introducing nitrogen gas. Elution was conducted with diethyl ether by passing through the cartridge. Eluted samples were dried with sodium sulfate and were distilled by a rotary evaporator. Dimethyl sulfoxide (DMSO) was used as redissolving solution for residues of distillation.

RESULTS

Fig. 1 shows dose-response curve of DNA strand breaks induced by ^{137}Cs γ-rays. ΔD represents the difference between the percent D of unirradiated cells and irradiated cells. There are some reports about the number of DNA strand breaks induced in mammalian cells by γ-rays irradiation (14-16). According to Loon et al (16) (2.5×10^{-10} single strand breaks / Gy / dalton), 1 Gy of γ-rays induce one DNA strand break per 10^7 base pair, and the horizontal axis of Fig. 1 represents the number of DNA strand breaks per 10^7 base pairs. Therefore, we use this Fig. 1 as a calibration curve of DNA strand breaks.

Different from ionizing radiation, many chemical carcinogens do not break DNA directly but form DNA base adducts. In this case, DNA strand breaks are induced when these adducts are removed by repair endonucleases in cells, but these strand breaks are rejoined rapidly. After exposure to these carcinogens, we incubated cells for 3 h in medium containing araC and

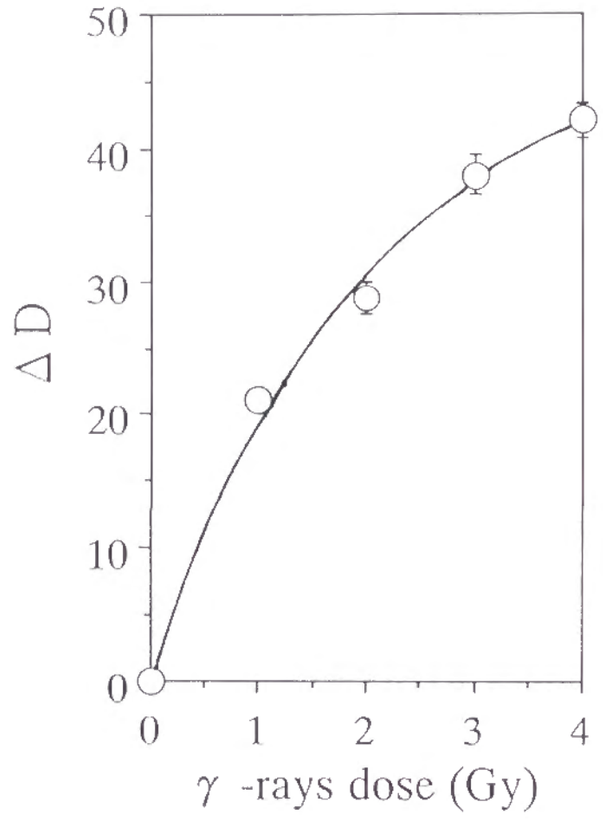
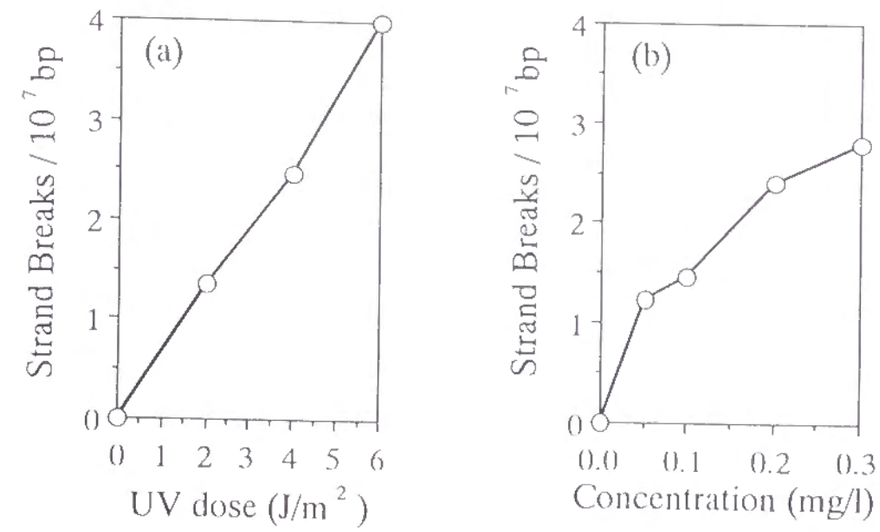


Fig. 1 Dose-response curve of DNA strand breaks induced by ^{137}Cs γ -rays. ΔD represents the difference between the percent D of unirradiated cells and irradiated cells. We can use this figure as a calibration curve of DNA strand breaks. The horizontal axis of this figure represents the number of DNA strand breaks per 10^7 base pairs.



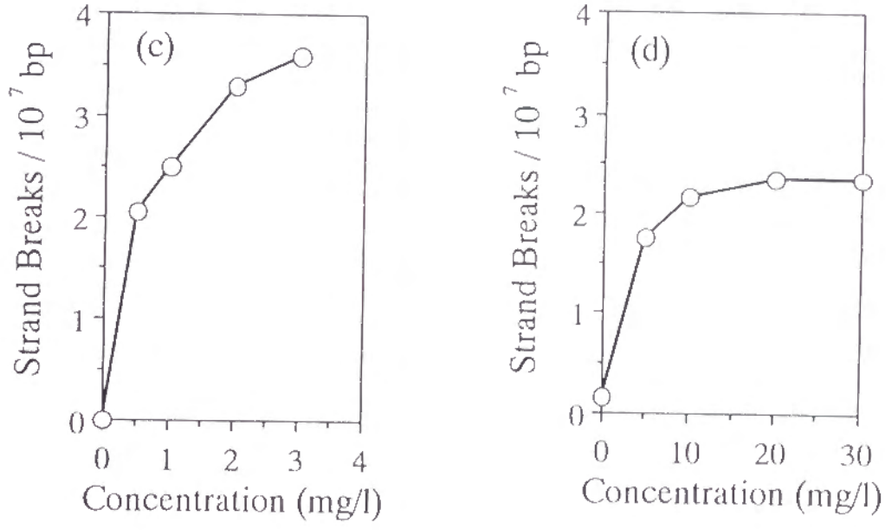


Fig. 2 DNA strand breaks induced by physical and chemical carcinogens, that is, (a): ultraviolet light, (b): 4NQO, (c): MNNG, (d): B(a)P. B(a)P was activated with S9-mix before exposed to human cells.

hydroxyurea to prevent the rejoining of DNA strand breaks. Fig. 2 shows the DNA strand breaks induced by UV-light, 4NQO, MNNG and B(a)P. Dose dependent increase in DNA strand breaks was observed in all the cases.

We concentrated the effluent of O-City waste water treatment plant. Human cells were exposed to the sample for 1 h and incubated in medium containing araC and hydroxyurea for 3 h. Fig. 3 shows the result of FADU assay. Dose dependent increase in DNA strand breaks was observed. The number of induced DNA strand breaks was 0.56 per 10^7 base pairs when the water sample was concentrated 200 fold.

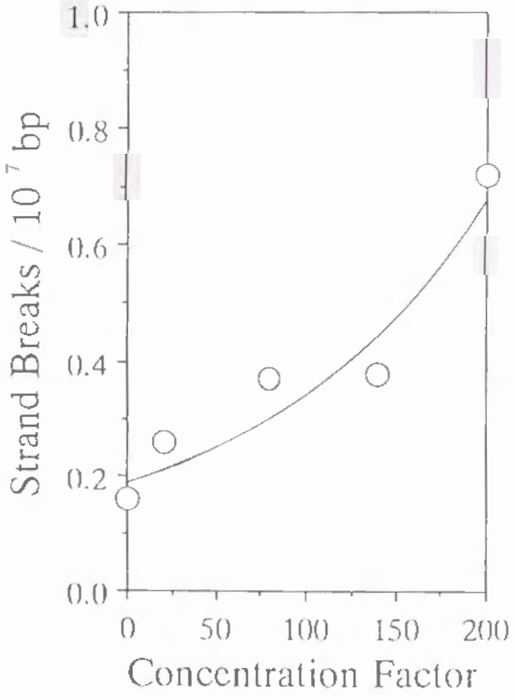


Fig. 3 DNA strand breaks induced by the effluent of O city waste water treatment plant.

DISCUSSION

We tried to apply the FADU to short-term bioassay. We used SV40-transformed human fibroblast cell line WI38-VA13 which is easy to maintain, and we modified the original protocol as described Birnboim and Jevcak (2). The number of cells we used for each experiment is 1/10 of that of original protocol, and the concentration of ethidium bromide in the solution E (6.7 μg/ml at the original protocol) is reduced to 2 μg/ml, and the time and temperature of incubation are changed.

We could detect DNA strand breaks induced by UV light, MNNG, 4NQO and B(a)P efficiently by using the modified protocol. The strand rejoining inhibiter, araC and hydroxyurea, are necessary for the sensitive detection of the DNA strand breaks, because no increase in DNA strand breaks was observed when cells were incubated in the normal medium for 3 h after 6 J/m^2 UV-irradiation (data not shown). In these cases, the number of strand breaks is almost the equal to the number of DNA adducts repaired. We should be noted that the number of strand breaks we observed did not include the number of remaining DNA adducts which are not repaired.

Strand breaks are also induced by the treated sewage of O. City. We concentrated hydrophobic organisms selectively from the effluent of waste water treatment plant. Hydrophobic organisms easily pass through the cell membrane, and tend to accumulate in the food chain. If these hydrophobic

organisms accumulate in cells of aquatic animals, serious DNA damage will occur in these animals.

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Chapter 3

Detection and Quantification of DNA Strand Breaks in Human Cells Induced by Contaminants in Japanese Tap Waters.

ABSTRACT

Activity of organic materials contaminating in tap water to induce DNA damage in human cells was investigated. Tap water samples were concentrated using the Sep-pak C_{18} , the CSP800 and the blue rayon and DNA strand breaks induced in human cells were measured by FADU assay. The tap water samples of Osaka City, concentrated by the Sep-pak C_{18} or the CSP800, induced dose dependent increase in DNA starnd breaks in human cells. Rat liver S9 fraction or glutathione or boiling inactivated the DNA toxicity of tap water samples.

INTRODUCTION

Lake Biwa and the Yodo River have a large basin area (7,281 km²) with the highly developed water complex where about 14 million people live. The Lake Biwa where the Yodo River flows out is a large natural reservoir with relatively good water quality for the purpose of drinking as well as much other utilization. However, in the middle of the basin, many urban and industrial wastewater and agricultural flumes flows into the river. Big cities such as Osaka and Kobe are located further downstream, and the cities take water from the Yodo River as a source of municipal water supply (Fig. 1). Our recent study using the *Bacillus subtilis* rec-assay showed that DNA damaging potential of contaminants in the river water increased as the river goes down (1), and these DNA toxic substances may contaminate drinking water. It is important to know the genotoxic effects of substances containing in tap water on human cells for estimation of the cancer risk to human beings.

MATERIALS AND METHODS

Concentration of Water Samples with Sep-Pak C_{18} Cartridge

Water samples (20 liters) were collected in plastic bottles. After taking the samples back to our laboratory, the water samples were adjusted

to pH 2 with H_2SO_4 and were passed through the Sep-Pak C_{18} cartridge (MILLIPORE, Milford, MA) (5 liters per cartridge) at room temperature at the flow rate of 4 ml/min. When adsorption was completed, 2 mL of pure water was passed through the cartridge to wash the resins and remaining water was purged away from the cartridge by introducing nitrogen gas. The adsorbed substance was eluted with 20 ml of diethyl ether by passing through the cartridge. Sodium sulfate was added to the eluted sample to dehydrate and the sample was distilled by a rotary evaporator. The residue was redissolved in 0.4 ml dimethyl sulfoxide (DMSO).

Concentration of water samples with CSP800 resin

The sample water were pretreated as described in the Sep-pak method. The water was passed through the glass column (15mmø × 80mm) containing 3ml of ion exchange resin CSP800 (MITSUBISHI KASEI, Tokyo, Japan) at room temperature. Ten liters of water was passed through per column at the filtration rate of 4 mL/min. When adsorption was completed, remaining water was purged away from the cartridge by introducing nitrogen gas. The adsorbed substance was eluted with 20ml of methanol by passing through the column. The eluted sample was distilled by a rotary evaporator. The residue was redissolved in 0.2 ml of dimethyl sulfoxide (DMSO).

Concentration of Polyaromatic Substances with Blue Rayon

Before the sample water was applied to the column, free available chlorine in the water was neutralized with sodium sulfite. The neutralized water (10 liters) was passed through a glass column (15mmø × 80mm) with the Blue Rayon (1 g) (FUNAKOSHI, Tokyo, Japan) at room temperature. When adsorption was completed, the Blue Rayon was washed once with pure water and excess moisture was removed with a paper towel. The absorbed substance in the Blue Rayon was then extracted with 200 ml of methanol-conc. ammonium water (50 : 1, v/v) with shaking for 30 min. The extraction was repeated once, and the solutions extracted each time were combined and dried with a rotary evaporator at 40 ℃. The residue was redissolved with 0.2 ml of DMSO.

Chemicals, Buffer Solutions and Cells

Composition of the solutions used was as follows. Solution A: 0.54mM EDTA in PBS; solution B: 9M urea, 10 mM NaOH, 2.5 mM trans-1,2-diaminocyclohexane-N,N,N',N' tetraacetic acid, 0.1% w.v sodium dodecylsulphate; solution C: Mixture of 0.425 volume solution A and 0.575 volume 0.2N NaOH; solution D: 1M glucose, 14mM mercaptoethanol; solution E: 2μg/ml ethidium bromide in 13.3 mM NaOH.

Rat liver 9,000 ×g supernatant fraction (S9) was obtained from Oriental

Yeast, Tokyo, Japan. Composition of the S9-mix used in this study was as follows, 1 mM HEPES buffer pH 7.2, 1 mM $MgCl_2$, 6.6 mM KCl, 0.8 mM NADP, 1 mM D-Glucose 6-phosphate, 4.4 %(v/v) of S9 (protein content; 22.78 mg/ml S9) in serum free MEM.

SV40 transformed human fibroblast cell lines was used . A normal human cell line WI-38-VA13 was obtained from the American Type Culture Collection, Rockville, MD. The cells were cultured in Dulbecco's modified minimum essential medium (Nikken, Kyoto, Japan) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT).

FADU Assay

Cells (1.1×10^6) were plated on 100 mm tissue culture dishes (IWAKI GLASS, Tokyo, Japan) and cultured in Dulbecco's modified minimum essential medium (MEM) (Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) for 18 hour at 37℃ in a CO_2 incubator. Each plates were washed once with Dulbecco's modified phosphate buffered salts solution (PBS) and the cells were exposed for 1 h to various concentration of samples in a serum free MEM at 37℃ in a CO_2 incubator. If the S9-mix activation was required, the chemicals were dissolved in S9-mix instead of the serum-free MEM. After exposure, each plates were washed once with PBS, and the cells were incubated for 3 h in MEM containing 10% serum,

10μM arabinofuranosyl cytosine and 2 mM hydroxyurea at 37℃ in a CO_2 incubator. Cells were washed with PBS, trypsinised, centrifuged and resuspended in 1.5 ml of solution A (8×10^5 cells/ml), and the FADU was carried out to estimate DNA strand breaks as described in Chapter 2.

Aliquots (0.2ml) of the cell suspension were placed into 2 sets of three15ml polystyrene tubes each labeled T, B or P, and according to treatment, in duplicate. Solution B (0.2ml) was added to all tubes which were incubated for 10 minutes on ice to ensure cell lysis and chromatin disruption. Solution D (0.4ml) was added with mixing to the tubes labeled T to prevent DNA unwinding. Solution C (0.2ml) was gently dispersed into all the tubes which were kept on ice for 30 minutes to initiate unwinding. The tubes labeled B were sonicated to ensure complete disruption of the DNA before all tubes were transferred to a water bath at 60 ℃ for 60 minutes for DNA unwinding under alkaline conditions (pH12.8). The tubes labeled P were transferred to a water bath at 28 ℃ for 45 minutes. DNA unwinding reaction in the tubes labeled P and B was arrested by the addition of 0.4 ml of solution D to bring the pH to 11.1. Solution E (1.5ml) was added to all tubes and the fluorescence was measured by a SHIMADZU RF-5000 spectrofluorometer (excitation, 520nm; analyzer, 590nm).

The percentage of double stranded (ds) DNA was determined from the fluorescence of the tubes labeled B (background fluorescence), T (total

fluorescence due to ds DNA and contaminants) and P (ds DNA remaining following unwinding in alkali) from the fillowing equation:

% ds DNA = (P-B)/(T-B)×100

Ames Salmonella /microsome assay

The bacterial strain used in the Ames Salmonella /microsome assay was *S.typhimurium* TA100. The assay was carried out by the liquid preincubation procedure (3).

E.coli Repair Test

The *E. coli* strains used in this assay were WP2 (wild type) and its repair-deficient derivertives WP2uvrA (uvrA⁻), ZA60 (recA⁻) and WP100 (uvrA⁻,recA⁻). The assay was carried out as described by nunoshiba et al (4). Briefly, Overnight culture of *E.coli* cells was diluted 1/10 with LB medium followed by further incubation for 3 h with gentle shaking. Cells were washed, resuspended in M9 buffer containing the tap water extract, and incubated for 60 min at 37 ℃ with gentle shaking. Viability was determined by colony formation on LB plates.

RESULTS

Genotoxicity of Tap Waters of Osaka City

The points and dates of sampling and methods of concentration were listed in table 1. This research was done from May 1993 to July 1994. The genotoxicity of tap water of Osaka City (May 1993, concentrated by the Sep-pak method) to bacteria or human cells was tested by Ames assay, *E. coli* repair-test and the FADU methods (Fig.2). Fig. 2(a) shows the result of the Ames assay. When the strain TA100 was treated with the concentrate of tap water of Osaka City, without S9 mix, dose-dependent increase of mutation rate was observed, but when the sample was activated by S9-mix, no increase of mutation rate was observed. Fig. 2(b) shows the result of the *E. coli* repair test. In comparison with the strain WP2 (wild type), the strain WP2-*uvrA* (*uvrA* deficient) and ZA60(*recA* deficient) were slightly sensitive, and the strain WP100(*uvrA*, *recA* double deficient) was extremely sensitive to the concentrate of tap water of Osaka City. Fig. 2(c) shows the result of the FADU assay. When human cells were exposed to the sample for 1 hour, and incubated with medium containing araC and HU for 3 hour, DNA strand breaks were induced dose-dependently. These results suggest that the tap water sample is genotoxic both in human and bacterial cells.

Table 1 Sampling points and concentration methods of tap waters

Date	Points	Methods
12 April 1993	Osaka City	Sep-pak C_{18}
1 May 1993	Osaka City	Sep-pak C_{18}
21 June 1993	Osaka City	Sep-pak C_{18}
21 June 1993	Kyoto City	Sep-pak C_{18}
23 June 1993	Kyoto City (groundwater)	Sep-pak C_{18}
18 October 1993	Osaka City	Sep-pak C_{18}, Blue Rayon
20 July 1994	Osaka City	CSP800, Blue Rayon

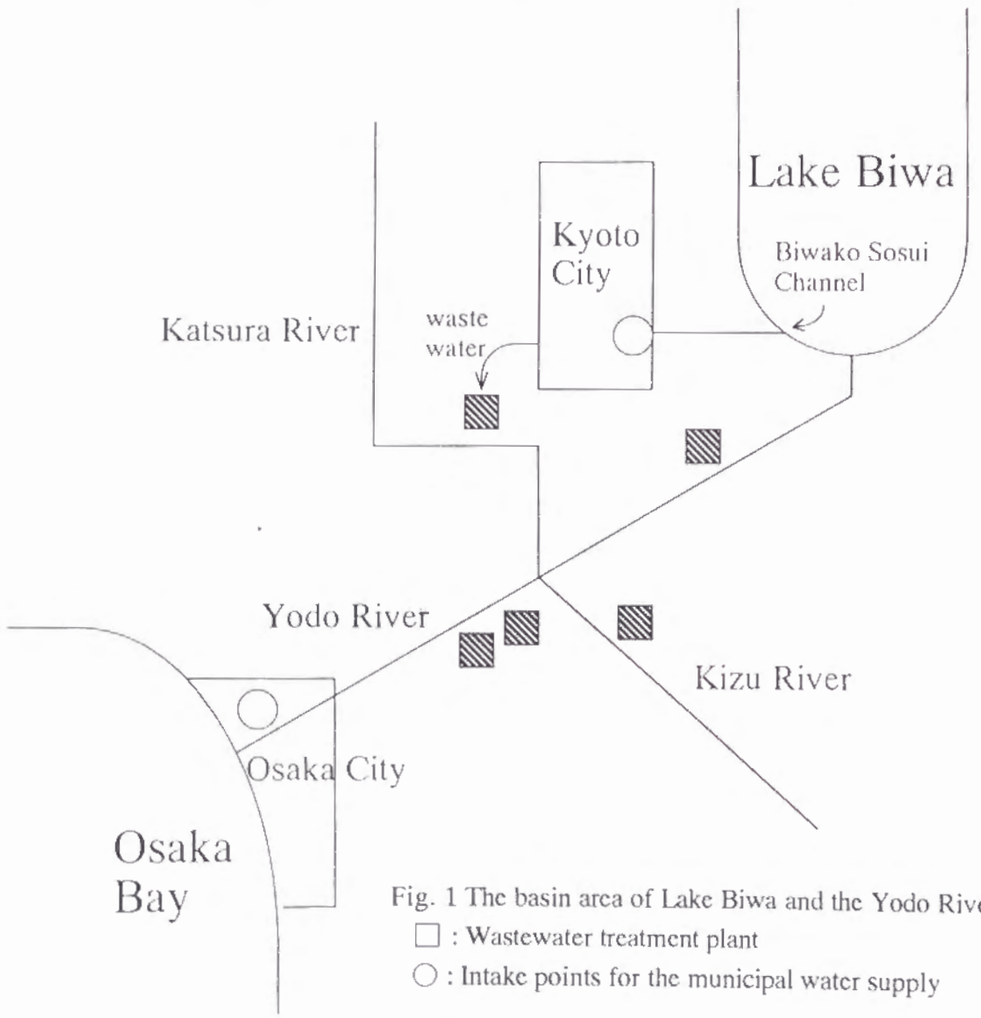
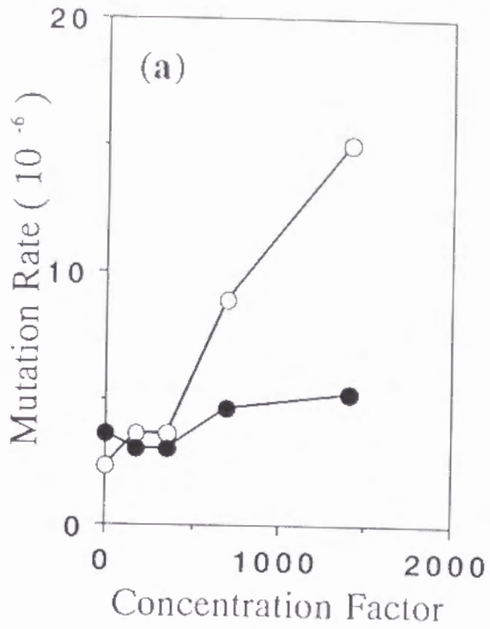
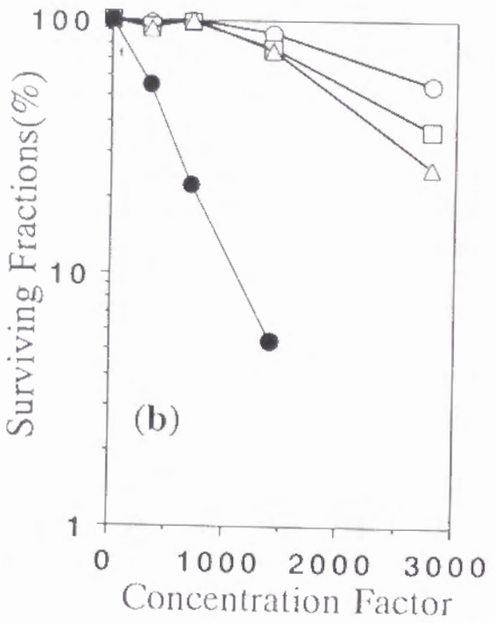


Fig. 1 The basin area of Lake Biwa and the Yodo River.
□ : Wastewater treatment plant
○ : Intake points for the municipal water supply





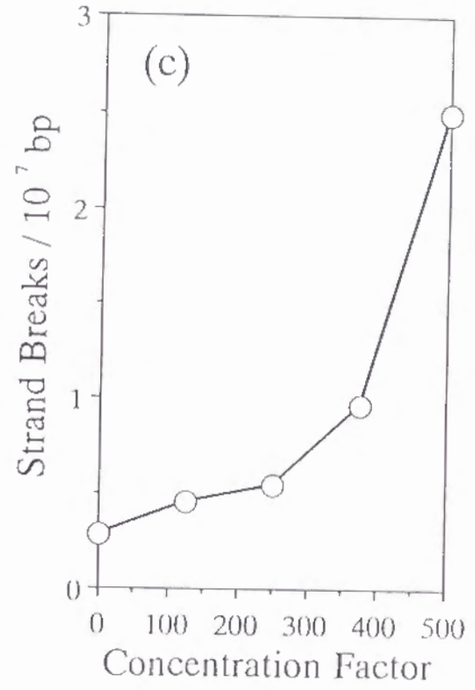


Fig. 2 Genotoxicity tests of tap water of Osaka City (May 1993).
Water sample was concentrated by the Sep-pak C_{18} method.
(a) Results of the Ames assay; Salmonella typhimurium TA100 was exposed to the water sample with (●) or without (○) S9 mix.
(b) E.coli Repair test; The E. coli strains used in this test are ○ :WP2 (wild type), □ : WP2-uvrA (uvrA deficient), △ : ZA60 (recA deficient), ● : WP100 (uvrA, recA double deficient).
(c) Result of the FADU assay.
The *E.coli* Repair test and the FADU assay were carried out without S9 mix.

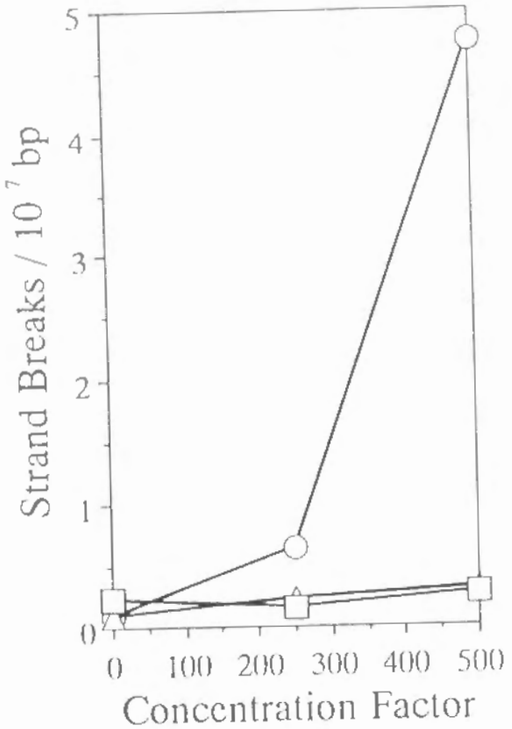


Fig. 3 DNA strand breaks induced by tap water of Osaka City (21 June 1993)(○), Kyoto City (21 June 1993)(△) and ground water of Kyoto City (23 June 1993)(□). Water samples were concentrated by the Sep-pak C_{18} method.

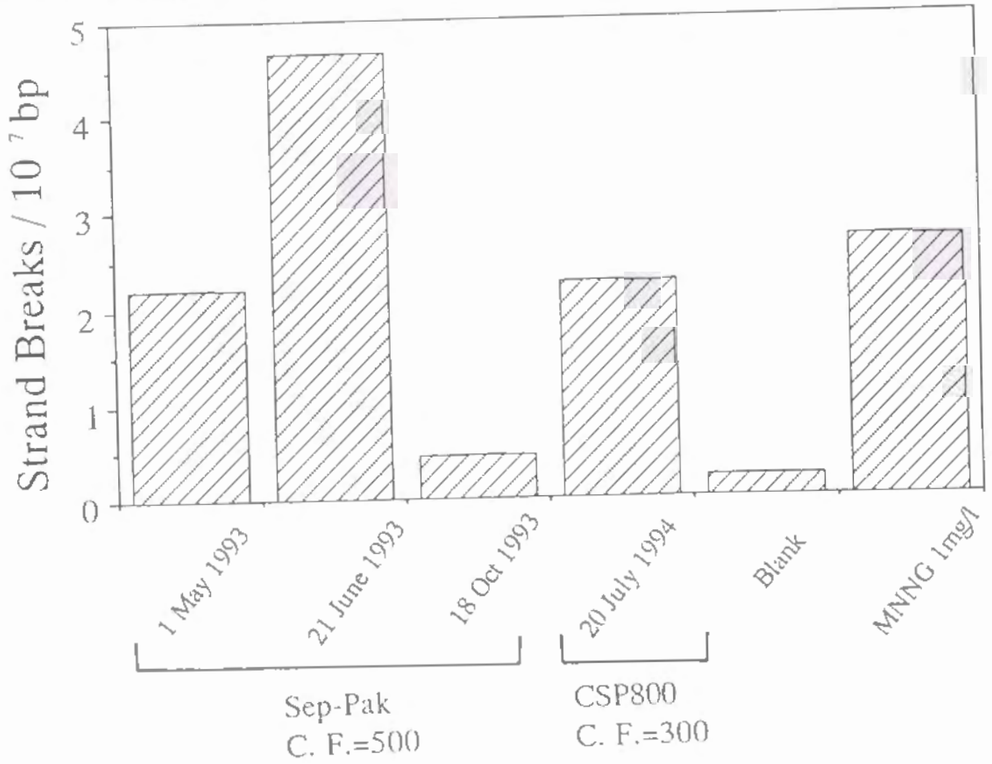


Fig. 4 The seasonal change of DNA strand breaks induced by tap waters of Osaka City. Water samples were concentrated 500 fold by the Sep-pak C18 method (May, June, October 1993) and 300 fold by the CSP800 method (July 1994).

We compare the DNA strand breaks induced by the Sep-pak concentrate of tap waters of Osaka City (21 June 1993), Kyoto City (21 June 1993) and ground water of Kyoto City (23 June 1993). The tap water of Osaka City had very high potential to induce DNA strand breaks compared to the tap water or ground water of Kyoto City which locates 30 km upstream from the Osaka City along the Yodo River. About 4.7 strand breaks per 10^7 base pairs were induced when cells were exposed to the 500 folds-concentrated water sample of Osaka City.

Fig.4 shows the strand breaks induced by tap waters of Osaka City, which were taken on different days and concentrated by Sep-pak or CSP800 methods. The numbers of induced strand breaks were changed by the day of sampling, but all the samples induced strand breaks more than 2-fold of those of the blank sample.

Contribution of the Polyaromatic Substances to the DNA Damaging Activity of Tap Waters.

We concentrated polyaromatic substances selectively using the blue-rayon. Fig. 5 shows the DNA strand breaks induced by tap waters of Osaka City which were concentrated by this method. The sample water which was taken on 18 October 1993 induces DNA strand breaks dose-dependently with or without S9 mix, but the sample which was taken on 20 July 1994 did not

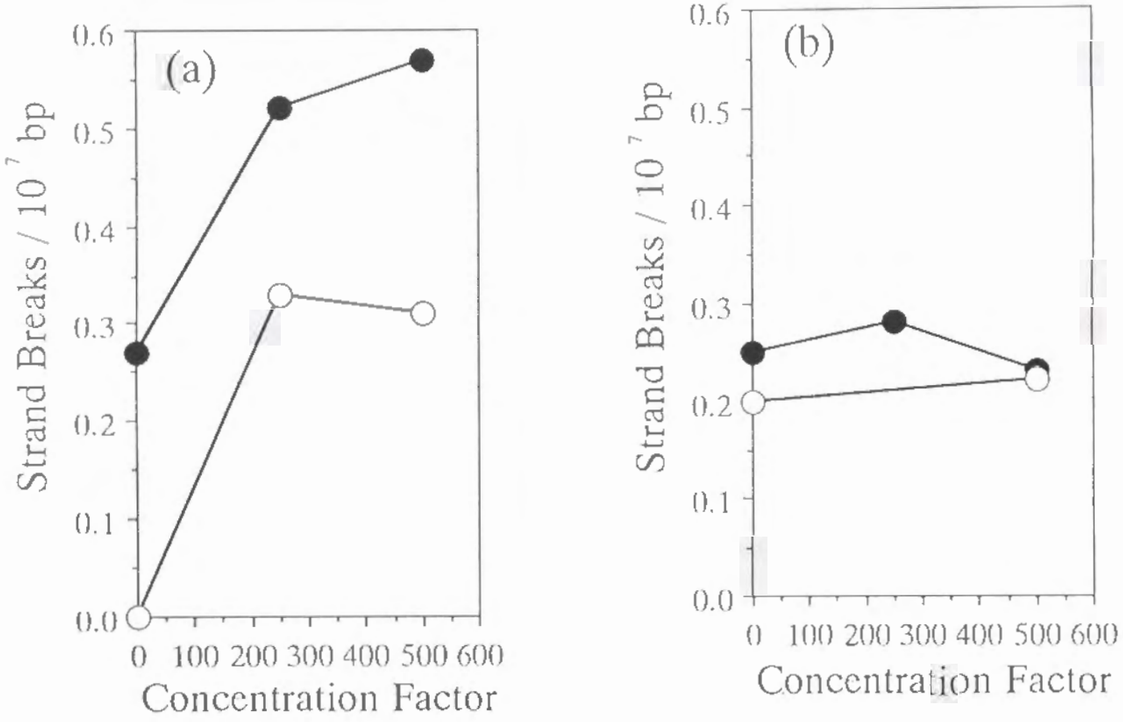


Fig. 5 DNA strand breaks induced by the blue rayon concentrates of the tap waters of Osaka City. (a): October 1993, (b): July 1994. The water samples were exposed to human cells with (open circle) or without (closed circle) S9-mix activation.

show dose-dependent increase in DNA strand breaks with or without S9 mix.

Detoxicate Factors against the DNA Damaging Agents in the Tap Water

To investigate the influence of S9 mix activation on induction of strand breaks, we took tap water of Osaka city on 18 October 1993 and concentrated the sample using the Sep-pak method, and DNA strand breaks were measured by the FADU method with or without S9 mix activation (Fig. 6). The sample induced dose-dependent increase in DNA strand breaks when cells were treated without S9 mix activation, but the potential to induce DNA strand breaks was completely vanished when cells were treated with the S9 mix.

To investigate the antigenotoxic activity of glutathione, the tap water sample (20 July 1994, Osaka City, concentrated by CSP800 method) was preincubated with 0.033mM or 0.33mM of glutathione for 10 min at 37 ℃ in the serum-free MEM prior to exposure of human cells, and DNA strand breaks were measured (Fig. 7). The number of the DNA strand breaks induced by the tap water sample was reduced by preincubation with glutathione.

Influence of Boil

The tap water sample (21 June 1993, Osaka City) was boiled for 1 min in

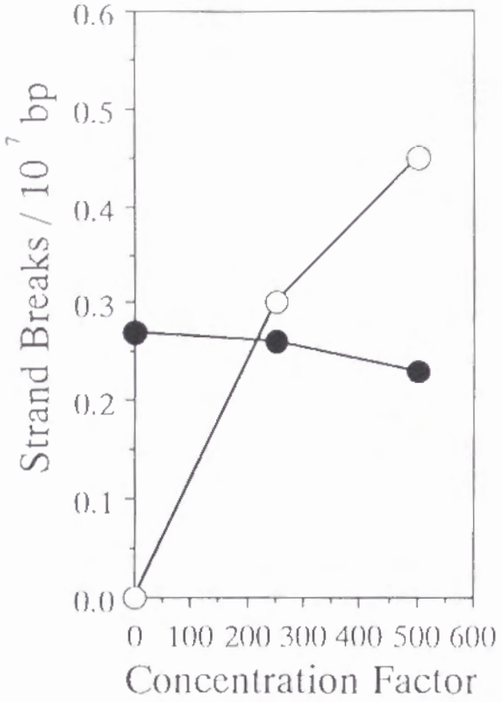


Fig. 6 DNA strand breaks induced by the Sep-pak C18 concentrate of the tap water of Osaka City (October 1993). The water samples were exposed to human cells with (open circle) or without (closed circle) S9-mix activation.

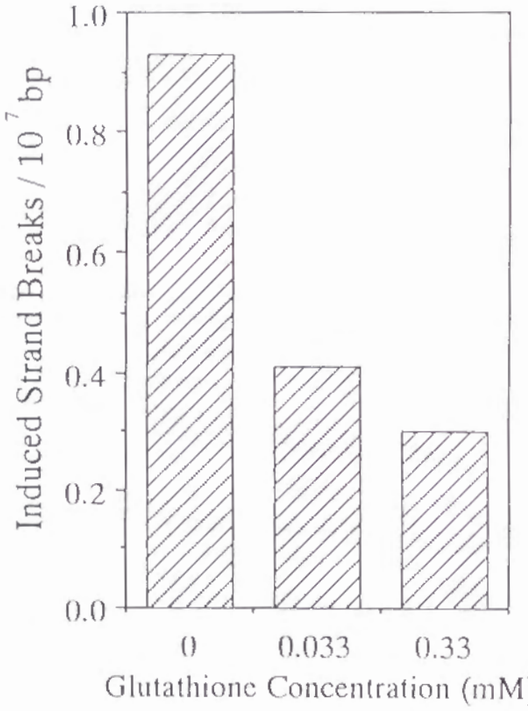


Fig. 7 Antigenotoxic effect of the glutathione. The tap water sample (Osaka City, July 1994, concentrated by the CSP800 method) was preincubated with 0.033mM or 0.33mM of glutathione for 10 min at 37 ℃ in serum free MEM prior to exposure to human cells. The S9-mix activation did not carried out.

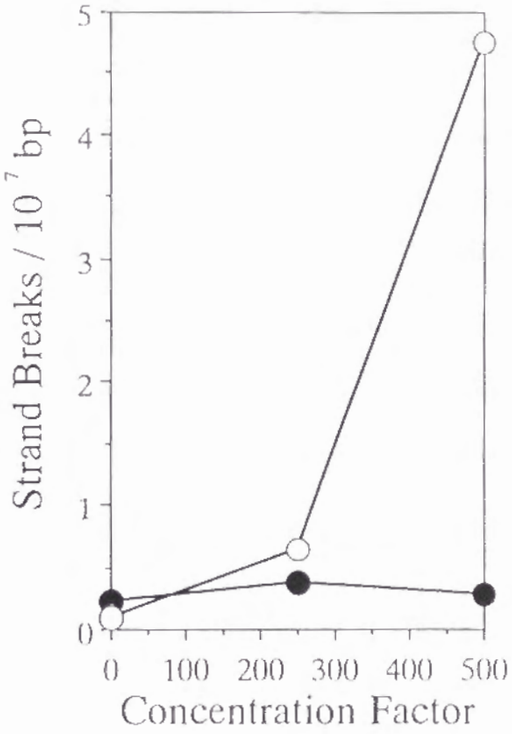


Fig. 8 Influence of boil. The tap water sample (June 1993, Osaka City) was boiled for 1 min (closed circle) or untreated (open circle) before it was concentrated by the Sep-pak C_{18} method. The FADU assay was carried out without S9-mix activation.

an aluminum kettle on a gas range, and concentrated by the Sep-pak method. DNA strand breaks measured by the FADU method were shown in Fig. 8. The potential to induce DNA strand breaks was dramatically reduced when water sample was boiled.

DISCUSSION

The tap water of Osaka City have potencial to induce DNA strand breaks in human cells, although the extracts studied may not be completely representative of the substances found in chlorinated water because the methods used to extract organic material from water were somewhat selective and would not result in equal concentration of all components; in particular, volatile substances may be lost.

The high DNA damaging activity of tap water of Osaka City compared to the that of Kyoto City may reflect the genotoxicity of the source of municipal water supply. This result is not contradictory to our previous findings(1). The water source of Kyoto City is transported directly from the Lake Biwa through the Biwako-Sosui channel, which is about 20 kilometer length open channel, and no waste flows in this channel, while the intake point for the water supply of Osaka City locates downstream the Yodo River, where many pollution sources flow in, that are treated sewage, industrial and

agricultural waste, and urban run off from most areas of Kyoto City.

Glutathione and rat liver S9 fraction reduce the potencial to induce DNA strand breaks of the tap water sample. It has been reported that Glutathione and rat liver S9 fraction reduce the mutagenicity of 3-Chrolo-4-(dichrolomethyl)-5-hydroxy-2(5H)-furanone (MX), which is estimated to account for about one-half to one-third of the total bacterial mutagenicity of the chlorinated tap water (5,6). These reports and our experiment did not clarify the contribution of MX on genotoxicity of tap waters, but one of the substances which cause DNA damage in human cells may be MX. Anyway, the genotoxic substnces containing in tap water may be inactivated by enzymes and glutathione in the human body.

Our data suggest that the human risk is also reduced when tap water is boiled. It is not due to the evaporation of low boiling point DNA damaging substances, because low boiling point substances are excluded during the process of Sep-pak method. It may be due to the heat denature or decomposition of the DNA damaging substances.

Our data directly demonstrate that the organic materials containing in tap water induce DNA damage in human cells, but mutagenicity and carcinogenicity is still unclear. Further study is necessary to estimate the actual risk of tap water to human health.

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Chapter 4

Molecular analysis of mutations induced by 2-chloroacetaldehyde, the ultimate carcinogenic form of vinyl chloride, in human cells using the improved shuttle vector system.

Abstract

Vinyl chloride (VC) is a carcinogen which is associated with induction of cancers in liver, brain, lung and haematolymphopoietic system. To study the VC mutagenesis at the molecular level in human cells, we treated the shuttle vector plasmid pMY189 with chloroacetaldehyde (CAA), the ultimate carcinogenic form of VC, and transfected the plasmid to human fibroblast cell lines. The plasmids pMY189 were constructed by modification of the plasmid pZ189 to be able to apply to the fluorecence dye primer cycle sequencing method. The plasmids containing mutations in the *supF* gene were detected with the indicator bacteria system, KS40/pKY241, and the mutations were determined by the automatic DNA sequencer. Base sequence analysis of 109 mutant plasmids showed that the major type of base substitution mutations were the G:C to A:T transitions and most of the remaining mutations were G:C to T:A transversions and A:T to G:C

transitions. 73% of single base substitutions were located at G:C pairs in 5'-AAGG-3' or 5'-CCTT-3' sequences, suggesting that these sequences are the major target of mutations, mainly, G:C to A:T transitions. This sequence specificity may be extrapolated to the estimation of contribution of VC to carcinogenesis in human beings.

Introduction

Vinyl chloride (VC) is one of the widely used raw materials in the polymer industries. Many epidemiological studies and case reports have demonstrated that VC is associated with induction of cancers in liver, brain, lung and the haematolymphopoietic system. The IARC working group classified VC as belonging to Group 1 (1,2), which represents the substances with carcinogenicity supported by sufficient evidence.

VC is metabolized by the cytochrome P450-dependent monooxygenases to 2-chloroethylene oxide, and then rapidly converted to 2-chloroacetaldehyde (CAA) in mammalian livers (3). CAA reacts with DNA bases *in vitro* , resulting in producion of four known cyclic adducts: 1,N^6-ethenoadenine (EA), 3,N^4-ethenocytocine(EC), N^2,3-ethenoguanine(N^2,3-EG), and 1,N^2-ethenoguanine(1,N^2-EG) (4). These cyclic adducts were detected in liver DNA in rats following exposure to VC (5,6), and have been shown to cause mutations (7-13).

Recently, mutations in *ras* genes involving G:C to A:T transitions in the second nucleotide at codon 13 in the c-Ki-*ras*-2 gene were detected in liver angiosarcomas in VC plant workers (14). This type of mutation could be specific to VC since the same type of mutation was found in bacteria exposed to VC (9,10,11,12,13,15). We intend to confirm whether this VC specific mutation arises in human cells.

The shuttle vector plasmids, pZ189 (16) and its derivatives pS189 (17) and pYZ289 (18) have been widely used in assessing the carcinogen-induced mutations in mammalian cells. Each type of the plasmid carries a bacterial suppressor tRNA gene, *supF*, as a target gene for mutagenesis. The*supF* gene is small enough (176 base pairs) to facilitate rapid sequence analysis in many samples. We constructed a new shuttle vector plasmid, pMY189, by modification of the plasmid pZ189 to make the plasmid sequence sutable for the fluorecence dye primer cycle sequencing method.

We treated the shuttle vector plasmid pMY189 with CAA, the ultimate carcinogenic form of VC, and transfected the plasmids to human fibroblast cells. The plasmids containing mutations in the *supF* gene were detected with the indicator bacteria system developed by Akasaka et al. (19), and the mutations were analyzed by the automatic DNA sequencing.

Materials and Methods

Chemicals

2-Chloroacetaldehyde(CAA), ampicillin, chloramphenicol, nalidixic acid, Isopropyl-β-D-thiogalactoside (IPTG) and 5-bromo-4-chloro-3-indoyl-β-D-garactoside (X-gal) were obtained from Wako Chemicals (Osaka, Japan). Restriction endonucleases and other enzymes for the molecular cloning were obtained from Takara Shuzo (Kyoto, Japan). QIAGEN plasmid-kit and Wizard™ Minipreps DNA Purification Systems were purchased from QIAGEN Inc. (Chatsworth,CA, USA) and Promega (Madison, WI, USA), respectively.

Cells.

SV40 transformed human fibroblast cell lines were used . A normal human cell line WI38-VA13 (20) was obtained from the American Type Culture Collection (Rockville, MD). DNA repair deficient XP2OS(SV) cells were previously established by us from a Japanese group A XP patient (21). All cells were cultured in Dulbecco's modified minimum essential medium (Nikken, Kyoto, Japan) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT).

Plasmid Construction.

The shuttle vector plasmid pZ189 was modified to be directly applied to the dye primer cycle sequencing method followed by automatic DNA sequencing by 370A DNA sequencer (Applied Biosystems, Foster, CA, USA) . Two 42-mer oligonucleotides were synthesized by 380B DNA synthesizer (Applied Biosystems). One containes the sequence of the -21M13 fluorescent dye-labelled universal primer (5'-TGTAAAACGACGGCCAGT-3') which is the commercially available (Applied Biosystems) (5'-GACGAATTCTGTAAAACGACGGCCAGTGAGCTCGAATTCTTG-3'). The other is its complimentary sequences, (5'-CAAGAATTCGAGCTCACTGGCCGTCGTTTTACAGAATTCGTC-3'). Both oligomers contain EcoRI restriction sequences (5'-GAATTC-3') at their 5'- and 3'- ends and a SacI restriction sequence (5'-GAGCTC-3'). Twenty μg each of these oligomers were annealed by heating and cooling in solution of 750 mM NaCl and 75mM Na_3citrate. After ethanol precipitation, the annealed fraction (double strand DNA) was digested with EcoRI, and inserted into the EcoRI site of pZ189 with T4 DNA ligase. The direction of the inserted oligomer was confirmed by SacI digestion and DNA sequencing. The modified pZ189 was named pMY189 (Fig. 1.).

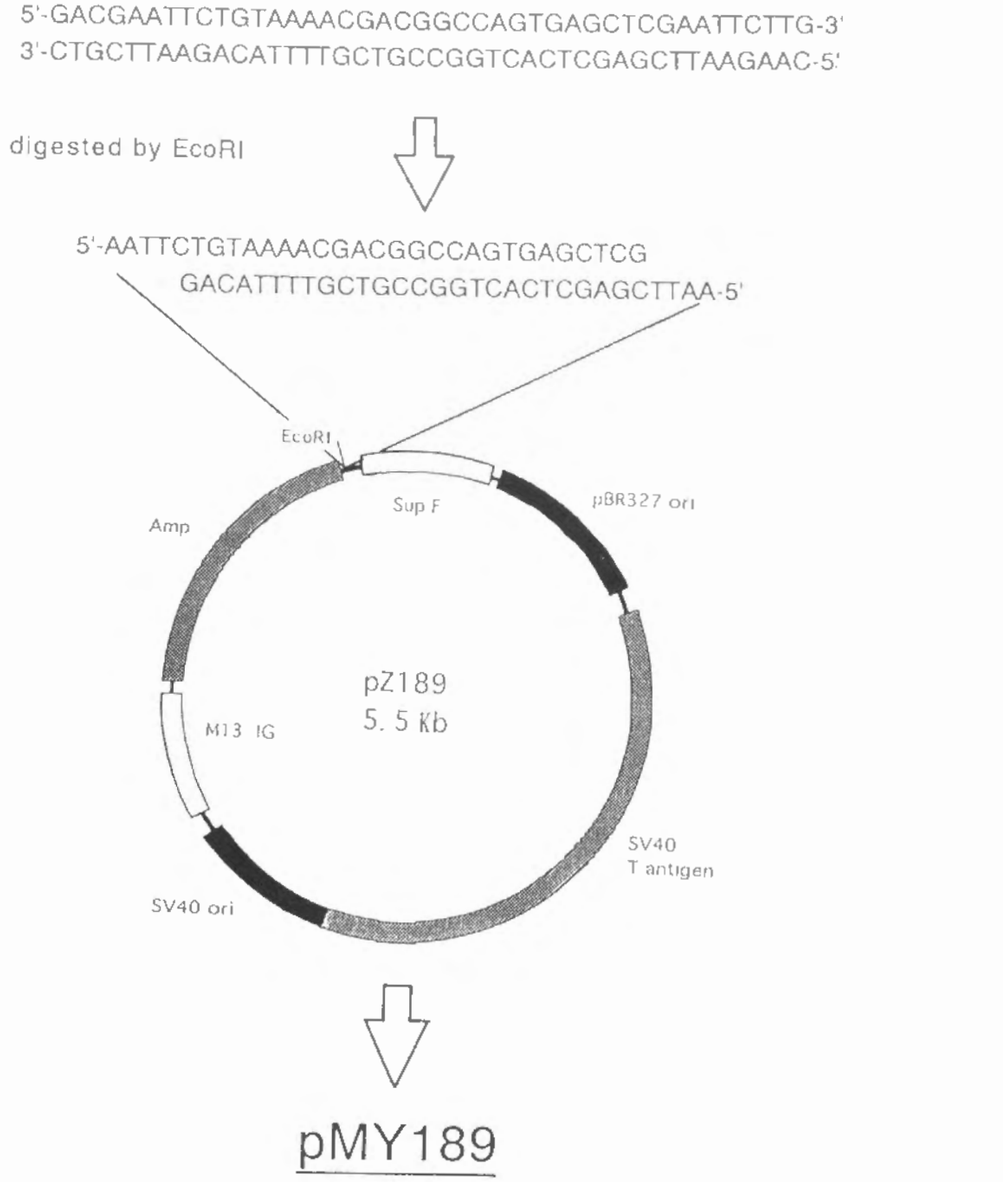

Fig. 1. Construction and the structure of the shuttle vector plasmid pMY189. Two 42 mer oligonucleotides, one contained a sequence of -21M13 5'-fluorescent dye-labelled universal primer for the automatic DNA sequencing, were annealed and digested by EcoRI, and ligated into the EcoRI site of the pZ189 .

Bacterial strains

The indicator *E.coli* strain KS40/pKY241(19) was kindly supplied from Dr. S. Akasaka, Division of Industrial Health, Osaka Prefectural Institute of Public Health, Osaka, Japan. KS40 is a nalidixic acid-resistant (*gyrA*) derivative of MBM7070 (*lacZ* (am) *CA7070 lacY1 HsdR HsdM Δ(araABC-leu)7679 galU galK rpsL thi*) (22), which has been used for detection of the mutated pZ189. Plasmid pKY241 was constructed by Dr.Akasaka et al (19) and contains a chloramphenicol resistant marker and a *gyrA*(amber) gene. *E.coli* KS40/pKY241 cells carrying active *supF* suppressor tRNA are sensitive to nalidixic acid, whereas the cells carrying mutated *supF* form colonies on plates containing nalidixic acid, chloramphenicol and ampicillin. IPTG and X-gal were further added to confirm selection of the mutated *supF* gene. *E.coli* cells containing active *supF* gene produce blue colonies, whereas the cells having mutated *supF* gene produce white or light blue colonies.

Treatment of Plasmids with CAA and Transfection to Human Cells.

Pure stocks of pMY189 were prepared by using the QIAGEN plasmid purification kit. The plasmids (40 μg) were treated with various concentrations of CAA in 0.3 M sodium acetate in total volume of 0.5 ml. The reaction was allowed to proceed for 1 hour at 37℃ followed by ethanol

precipitation of the plasmids to remove the nonreacted excess CAA, and the plasmids were redissolved in 0.5 ml of TE buffer (pH8) .

The human cells, WI38-VA13 or XP2OS(SV) were trypsinized, washed, and suspended in Dulbecco's phosphate-buffered saline (PBS) solution (pH 7.4-7.6). Cells (2×10^7) plus 14.4 μg CAA-treated pMY189 in PBS solution (0.2 ml) were placed in an electroporation chamber (electrodes 0.3 cm apart) (PDS, Inc., Madison, WI) and the cells were transfected with the plasmids by electric pulses (600 V, 5 times). The cells were plated in five 10-cm dishes and incubated at 37℃ for 72 h in a CO_2 incubator.

Plasmid Recovery, Selection of Mutated *supF*, and DNA Sequencing.

Plasmids were extracted from the cells using Wizard™ Minipreps DNA Purification Systems (Promega, Madison, WI, USA). The purified plasmids were digested with the restriction endonuclease DpnI (Boeringer-Yamanouchi, Tokyo, Japan) to eliminate the nonreplicated plasmids which retain the bacterial methylation pattern.

Plasmid DNA was introduced into the indicator bacteria KS40/pKY241 by the electro transformation apparatus E.coli Pulser (Biorad, CA., USA). The bacteria were plated on LB agar plates containing nalidixic acid, ampicillin, chloramphenicol at concentrations of 50 μg/ml, 150 μg/ml and 30 μg/ml, respectively, supplemented with IPTG and X-gal to select the

plasmids containing the mutated *supF* genes. A part of the bacteria was also plated on LB agar plates containing ampicillin and chloramphenicol to measure the total number of transformants. After 24h incubation at 37 ℃, colonies were counted and mutation frequencies were calculated.

Mutated plasmids were extracted and purified from the overnight culture with the Wizard Minipreps Purification System (Promega) and the base sequences of the *supF* gene of the plasmids were determined with the -21M13 primer and Dye-Primer Cycle Sequencing reagent Kit using 370A automatic DNA sequencer (Applied Biosystems).

Results

Plasmid Mutagenesis.

CAA treatment of the pMY189 plasmids increased the frequency of mutation in *supF* gene in both repair-proficient and repair deficient cells (Table 1). The background plasmid mutation frequency was 2.2 and 1.4×10^{-4} with VA13 and XP-A cells, respectively. The mutation frequency increased similarly in both cell lines following treatment of the plasmid with CAA; about 7- and 40-fold increase with 0.13M and 0.51M CAA, respectively.

Base Sequence Analysis

Analysis of 109 *supF* mutant plasmids transfected to the repair proficient cells was carried out by the nucleotide sequencing. Base sequence changes in plasmids were classified as single base substitutions, tandem base substitutions, multiple base substitutions (2 ≤ base substitutions more than 3 bases apart), frameshifts (single base insertions or deletions), and large deletions (Table 2). Large deletions (average deletion size, 113 base pairs; range, 26 to 184 base pairs) were found in 10% of the plasmids, and 12% of the plasmids contained the multiple base substitutions. The tandem base substitutions and the frameshift mutations

Table 1. Mutation frequency of 2-chloroacetaldehyde-treated plasmids pMY189 propagated in normal or xeroderma pigmentosum fibroblasts. Average colony numbers from 3 independant experiments are given with the standard error of the mean.

	WI38-VA13			XP2OS(SV)		
	No. of colonies		Mutation	No. of colonies		Mutation
Concentration of CAA(M)	Mutant	Total ($\times 10^4$)	frequency ($\times 10^{-4}$)	Mutant	Total ($\times 10^4$)	frequency ($\times 10^{-4}$)
0	85.0± 7.1	38.0	2.2±0.2	2.3± 1.9	1.6	1.4±1.2
0.13	151.3±11.9	10.2	14.9±1.2	160.7±18.3	12.1	13.3±1.5
0.26				241.0±23.8	7.7	31.4±3.1
0.51	989.0±61.7	16.7	59.2±3.7	154.0± 4.5	2.8	55.3±1.6

Table 2. Types of mutations in the supF gene in 2-chloroacetaldehyde -treated shuttle vector prasmids pMY189 propagated in human fibrobrast cells WI38-VA13

Type of mutation	No.	% of total
Base substitution		
single base substitution	79	72.5
tandem base substitution	1	0.9
multiple base substitution	14	11.9
Frameshift		
single base insertion	0	0.0
single base deletion	1	0.9
Deletion over 3bp	12	10.1
Others	2	1.8
Total	109	100

Table 3. Types of 2-chloroacetaldehyde-induced single base substitutions in the supF gene in shuttle vector plasmids pMY189 propagated in human fibrobrast cells WI38-VA13

Type of mutation	No.(%) of mutants	No. of mutants /target site[a](%)
Substitution of G:C	71 (89.9)	1.291 (86.3)
G:C to A:T	43 (54.4)	0.782 (52.3)
G:C to T:A	23 (29.1)	0.418 (27.9)
G:C to C:G	5 (6.3)	0.091 (6.1)
Substitution of A:T	8 (10.1)	0.205 (13.7)
A:T to G:C	7 (8.9)	0.179 (12.0)
A:T to T:A	1 (1.3)	0.026 (1.7)
A:T to C:G	0 (0.0)	0 (0.0)
Total	79 (100)	1.496 (100)

[a]Numbers of targets of the single base substitution mutations in the supF gene are 55 at G:C pairs and 39 at A:T pairs.

were scarce. The rest (73%) contained the single base substitutions.

Among the mutant plasmids with single base substitutions, 90% were the substitutions of G:C base pair (Table 3). The most frequent type of the base substitution mutation was G:C to A:T transition (54%). Other types of the base substitution mutation were G:C to T:A transversion (29%), G:C to C:G transversion (6%), A:T to G:C transition (9%) and A:T to T:A transversion (1%). No single base A:T to C:G transversion was detected. Figure 2 shows the possible targets of the single base substitutions which inactivate the suppressor function in the *supF* gene, compiled from the previous reports (16,17, 23-39). Among the possible 94 taget sites in the *supF* gene, 55 sites are present in G:C base pairs and 39 sites are present in A:T base pairs. Proportion of the base substitution at G:C base pairs is higher than that at A:Tbase pairs, even the ratio of G:C to A:T was normalized (Table 3).

Mutation Spectra.

Distribution of the single base substitutions, and tandem or multiple base substitutions in the *supF* gene with the repair proficient cell is shown in Fig. 2. Eight sites (123,133,134,156,159,160,168 and 169) had four or more single base substitutions. Most of the single base substitutions (91%) were produced at these hotspots. Seven of the 8

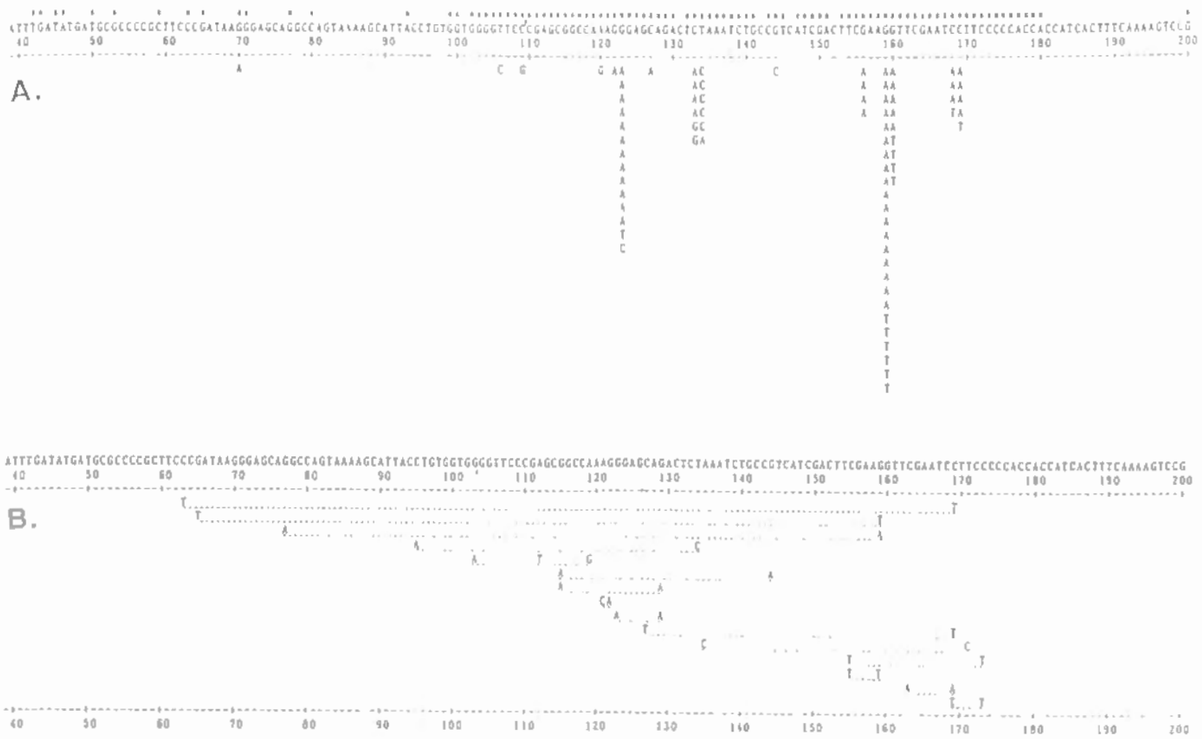

Fig. 2. Locations of the single (A), and tandem and multiple (B) base substitution mutations found in the *supF* suppressor tRNA gene in CAA-treated plasmids pMY189 propagated in WI38-VA13 cells. Each letter represents a single base substitution mutation found in an independent plasmid (A). Tandem and multiple base substitutions occuring in a clone are underlined (B). The possible targets of the single base substitutions which inactivate the suppressor function are as follows (Position (Reference)) : 42(16), 43(16,39), 45(16), 46(16), 50(16, 38), 53(28), 59(39), 62(39), 63(16), 65(16,24,26), 70(our data), 71(16,26), 77(16,31), 80(24), 93(24,26), 99(24,26,34), 100(16), 102(16,17,25), 103(16,17,25,37), 104(16,17,33,37), 105(16,17,37,33,38), 106(16,34,38), 107(37), 108(16,23,26,31,36-39), 109(16,17,24-26,29,31,33,34,37), 110(16,25,30,31,36), 111(16,23,26,30,37), 112(16,17,29,30,33,34,37,38), 113(16,17,23,25,26,31,34,37,38), 114(16,26), 115(16,17,25,29,37,38), 116(16,17,25,26,29,31), 117(16,36), 118(16,24,34), 119(16,34), 120(16,24,31,33-35,37,38), 121(27,37), 122(16,25,31,34,36,37), 123(16,17,23-26,32-34,37-39), 124(16,17,24,33,34,36,38,39), 125(16,37), 126(16,37), 127(16,17,25,29,33,34,37,38), 128(30), 129(16,17,24-26,31,32,34,35), 130(37), 132(16,34,37), 133(16,17,24-27,29,31-34,38), 134(16,27,31,33,34,37-39), 135(16,27,31,33-35,38,39), 136(16,17,24,26,27,29,31,33-37,39), 137(16,17,31,33,34), 138(37), 139(16,17,24-26,29,33-35,37,38), 140(16,24,25,30,34), 141(16,25,33,34,37,38), 143(16,25), 144(16,17,24,26,29,33,34,37), 145(37), 147(35), 148(Akasaka et al., in press), 149(26,33,35), 150(16,31,37), 151(37), 153(37,39), 154(16,31,35,37), 155(16,17,23,25,26,29,31-37,39), 156(16,17,23-26,31-39), 157(37), 158(16,31,34,37), 159(16,17,23-26,29,31-39), 160(16,17,23-27,29,30,31,33,34,38), 161(16,27,33,34,37), 162(16,25,27,34,36,37), 163(16,31,32,37), 164(16,17,24-26,29,31,33,34,36,37,39), 165(16,25,31,33,34,36,37), 166(27,37), 167(16,37), 168(16,17,23-27,29-39), 169(16,17,23-26,29-31,33-39), 170(37), 171(16,34,37,39), 172(16,17,24-26,29,33,34,37,38), 173(16,34,39), 174(16,17,25,37), 175(16,31,33,37), 176(17), 177(16,17,34,35), 178(17,25), 179(16,34), 180(37), 200(16). The supF promoter sequence starts at base pair 24, the pre-RNA sequence starts at base pair 59 ,the tRNA coding sequence starts at base pair 99 and the 3' flanking region starts at base pair 184 and ends at base pair 200.

hotspots were located at G:C base pairs. Five G:C to A:T or G:C to T:A hotspots (123,159,160,168 and169) are located at G:C base pairs in 5'-AAGG-3' sequences.

Discussion

Plasmids pMY189 treated with 2-chloroacetaldehyde (CAA) yielded the same frequency of mutations when they were propagated in XP-A and normal cells, suggesting that the DNA damage induced by CAA is not repaired by the nucleotide excision repair passway in human cells. This was supported by the preliminary experiment indicating that the survival of *E.coli* MBM7070 (wild type) and KY46 (uvrA) transformed by the CAA-treated pMY189 plasmids were same (data not shown). CAA induced DNA adducts were reported to be repaired by a human 3-methyladenine DNA glycosilase (40, 41), suggesting that DNA repair other than the nucleotide excision type may be involved.

Mutation spectra of the single base substitutions showed a marked feature. Among 79 single base substitutions, 72 (91%) were located in the eight mutational hotspots, even though there are 115 possible mutation target sites in the *supF* gene. There are four 5'-AAGG-3' or 5'-CCTT-3' sequences in the *supF* gene (positions 68-71, 120-123, 157-160 and 168-171). Fifty eight out of 79 (73%) single base substitutions were located at G:C pairs in these 5'-AAGG-3' or 5'-CCTT-3' sequences. This suggests that G:C pairs in 5'-AAGG-3' or 5'-CCTT-3' sequences are the major mutational targets by CAA. The possibility that we isolated sibling mutants is not likely, because we took 109 colonies out of about 3000 mutant colonies, and

no identical mutation was found in the tandem and multiple base substitutions, frameshifts and deletion mutations.

G:C to A:T transition mutation is predominant (50.9%), supporting the previous findings that chloroethylene oxide-treated *E.coli* (15) and CAA-treated gapped duplex M13 DNA (13) sustained mainly G:C to A:T transitions.

The adducts which can cause mutations in G:C base pairs would be the 3,N^4-ethenocytocine(EC) and the N^2,3-ethenoguanine(N^2,3-EG). The N^2,3-EG : T mismatch caused G:C to A:T transition in an *in vitro* DNA replication experiment (8). Site specifically incorporated N^2,3-EG in the M13 double strand DNA induced G to A transitions (11), and site specifically incorporated EC in the M13 single strand DNA (9) or gapped duplex DNA (10,12) induced mainly C to T mutations. These reports and our experiment could not clarify which adduct is the major contributor to mutations in human cells, but both EC and N^2,3-EG can induce G:C to A:T transitions.

The previously published data of the mutations in G:C base pairs induced by CAA or its related cyclic adducts are summarized in Table 4. Compared with other studies using M13 phage, our results shows relatively high frequency of G:C to T:A transversion mutations. It may reflect the different mechanism of the mutation fixation in *E.coli* and human cells, since the similar difference was observed in UV-induced mutations in M13 and human cells (33,42).

The published data of the mutations in A:T base pairs induced by CAA or its related cyclic adducts are summarized in Table 5. The predominance of A:T to G:C transition mutations in our data supported the previous findings that site-specifically incorporated EA and 4-amino-5-(imidazole-2-yl)imidazole (β) in single strand M13 DNA induced mainly A:T to G:C transition(9) in *E.coli*. The data from mutations of CAA-treated M13 gapped duplex DNA replicated *in E.coli*, however, showed the predominance of A:T to T:A transversions (13). In addition to the difference of the mechanism of mutation fixation in *E.coli* and human cells, the contradicting results with M13 may be due to the difference in the sites of the adducts produced in DNA. The adducts are located in the single stranded region of the gap in the gapped duplex DNA.

The shuttle vector plasmid pMY189 designed to be applied to the fluorescent dye primer cycle sequencing by an automatic DNA sequencer, made the analysis of the nucleotide sequences of the mutant *supF* genes easier and faster than the conventional radioisotope labelling and autoradiography method. This method also made the analysis more accurate than the dye deoxy terminator cycle sequencing method using the automatic DNA sequencer we previously used (33).

In conclusion, we found that about a half of the CAA-induced mutations were G:C to A:T transitions and that the most of the remaining

Table 4. Types of G:C base substitution mutations induced by the 2-chloroacetaldehyde or its related cyclic adducts, in comparison with other studies.

	No. (%) of mutants				
	EG[a] M13 dsDNA in *E.coli*	EC[b] M13 ssDNA in *E.coli*	EC[c] M13 gdDNA in *E.coli*	CAA treated[d] M13 gdDNA in *E.coli*	Our Data[e]
G:C to A:T	134(99.3)	31(56.4)	(64.0)	80(72.1)	62(64.6)
G:C to T:A	0 (0)	12(21.8)	(10.9)	20(18.0)	29(30.2)
G:C to C:G	1 (0.7)	0 (0)	(0)	11(9.9)	5(5.2)
1base deletion	0 (0)	12(21.8)	(25.0)	0 (0)	0 (0)
Total	135(100%)	55(100%)	(100%)	111(100%)	96(100%)

[a]Cheng,K.C. et al.(11); Site-specifically incorporated N^2,3-ethenoguanine (5'-GG(*E*G)AAA-3') in double strand M13DNA was replicated in *E.coli* cells.
[b]Basu,A.K. et al.(9); Site-specifically incorporated 3,N^4-ethenocytosine (5'-TAG(*EC*)GGG-3') in single strand M13DNA was replicated in *E.coli* cells.
[c]Palejwala,V.A. et al.(10); Site-specifically incorporated 3,N^4-ethenocytosine (5'-TT(*EC*)TT-3') in gapped duplex M13DNA was replicated in *E.coli* cells. Mutation was analyzed by the multiplex sequence analysis.
[d]Jacobson,J.S. et al.(13); The CAA treated gapped duplex M13DNA was replicated in UV-irradiated *E.coli* cells.
[e]Data contains all single, tandem and multiple base substitutions.

Table 5 Types of the A:T base substitution mutations induced by the 2-chloroacetaldehyde or its related cyclic adducts, in comparison with other studies.

	No. (%) of mutants			
	EA[a] M13 ssDNA in *E.coli*	β[b] M13 ssDNA in *E.coli*	CAA treated[c] M13 gdDNA in *E.coli*	Our Data[d]
A:T to G:C	17(56.7)	11(68.8)	4(17.4)	10(76.9)
A:T to T:A	5(16.7)	2(12.5)	18(78.3)	2(15.4)
A:T to C:G	8(26.7)	3(18.8)	1(4.3)	1(7.7)
Total	30(100%)	16(100%)	23(100%)	13(100%)

[a]Basu,A.K. et al.(9); Site-specifically incorporated 1,N^6-ethenoadenine (5'-GCT(*EA*)GC-3') in single strand M13DNA was replicated in *E.coli* cells.
[b]Basu,A.K. et al.(9); Site-specifically incorporated 4-amino-5-(imidazol-2-yl)imidazole(β) (5'-GCT(β)GC-3') in single strand M13DNA was replicated in *E.coli* cells.
[c]Jacobson,J.S. et al.(13); The CAA treated gapped duplex M13DNA was replicated in UV-irradiated *E.coli* cells.
[d]Data contains all single, tandem and multiple base substitutions.

mutations were G:C to T:A transversions and A:T to G:C transitions in the shuttle vector plasmid pMY189 propagated in human cells. G:C pairs in 5'-AAGG-3' or 5'-CCTT-3' sequences are the major target of mutations, mainly, G:C to A:T transitions. This sequence specificity can be extrapolated to the estimation of contribution of VC to carcinogenesis in human beings.

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Chapter 5

Specific Tandem CC → AA Base Substitutions Induced by Acetaldehyde

ABSTRACT

Acetaldehyde is present in tobacco smoke and is produced by the oxydation of ethanol and is confirmed to cause cancers in the respiratory organs in animals(1). Base sequence analyses of 102 mutant *supF* suppressor tRNA gene induced by acetaldehyde revealed that 63 % of the mutated *supF* genes had tandem base substitutions. The most predominant type of the mutations are CC → AA (GG → TT) transversions, which have rarely been reported previously. As the treatment with acetaldehyde yields very specific CC → AA tandem base substitutions in DNA, the base changes may be used as a probe to identify acetaldehyde as the causal agent in human tumors.

INTRODUCTION

Acetaldehyde is one of the most commonly used organic substances in industry as the raw material for the products such as acetic acid, dyes, photographic chemicals, antioxidants, plastics and synthetic rubber(2). Presence of acetaldehyde in many foods and automotive exhaust gases has been detected(1). Cigarette smoke contains 0.8-1.4 mg acetaldehyde as the major component(3). Human exposure to acetaldehyde may also be common as it is produced by the oxydation of ethanol. Acetaldehyde produces interstrand cross-links in calf-thymus DNA *in vitro* (4), and induces sister-chromatid-exchanges in bone-marrow cells in rodents(5,6) exposed *in vivo* and in cultured human lymphocytes(7). It also induces chromosomal aberrations in rat embryos(8), and mutations in cultured human skin fibroblasts(9). Inhalation of acetaldehyde causes adenocarcinomas and squamous-cell carcinomas in the nasal mucosa in rats and laryngeal carcinomas in hamsters(1).

RESULTS AND DISCUSSION

The shuttle vector plasmid pMY189, previously constructed by us (manuscript submitted), was exposed to various concentrations of acetaldehyde. The plasmid has the *supF* suppressor tRNA gene as an indicator of mutation and can replicate in both human and *Escherichia coli* cells. Acetaldehyde-treated shuttle vector plasmids were transfected into the human fibroblast cells,

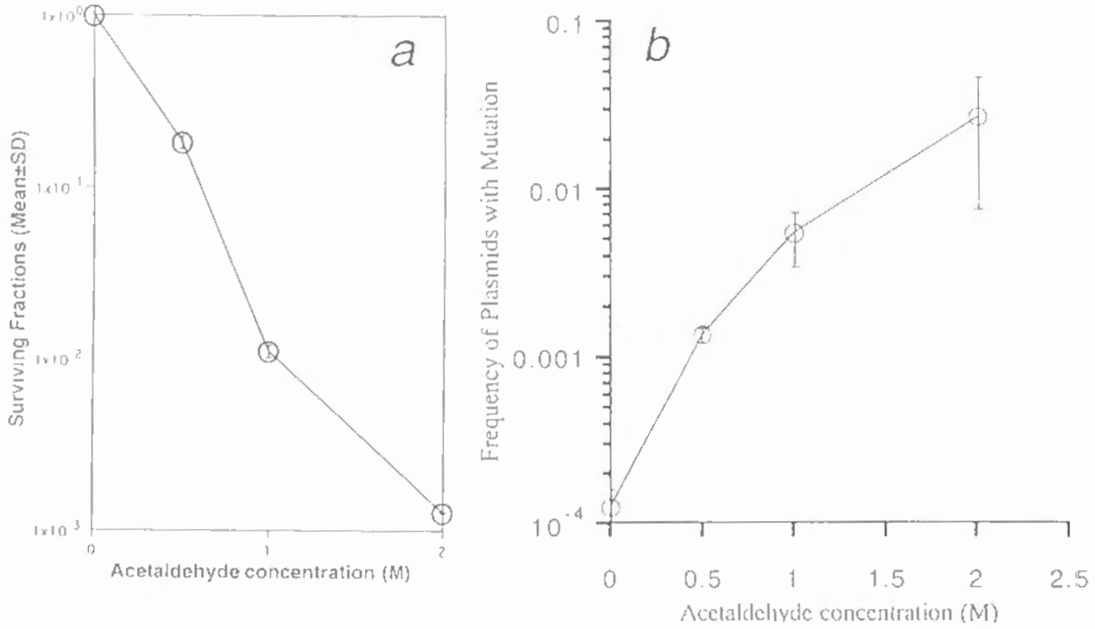

Fig. 1. a, Survival of acetaldehyde-treated pMY189 replicated in WI38-VA13 cells. The relative number of ampicillin and chloramphenicol-resistant bacterial colonies obtained after repair and replication of acetaldehyde-treated pMY189 in the cells followed by transformation of the indicator E.coli is shown. b, The frequencies of acetaldehyde-induced mutations in pMY189 replicated in WI38-VA13 cells. The frequency was calculated by the number of white bacterial colonies on LB agar plates containing ampicillin, chloramphenicol, nalidixic acid, IPTG and X-gal divided by the number of bacterial colonies on plates containing ampicillin and chroramphenicol.

METHODS. Plasmids pMY189 (80 μ g/ml in TE buffer (10mM tris,1mM EDTA)) were treated with various concentrations of acetaldehyde. The reaction was allowed to proceed at 37℃ for 1 hour followed by ethanol precipitation of the plasmids to remove nonreacted excess acetaldehyde, and the plasmids were redissolved in TE buffer (pH8). The human cells (WI38-VA13) (2×10^7) were transfected with the 14.4 μ g acetaldehyde-treated pMY189 by electric pulses. The cells were incubated at 37℃ for 72 h in a CO_2 incubator. The plasmid was extracted from the cells and digested with the restriction endonuclease DpnI to eliminate nonreplicated input plasmids with the bacterial methylation pattern. Plasmid DNA was introduced into the indicator bacteria *E.coli* KS40/pKY241(23) by electro transformation. The bacteria were plated on LB agar plates containing nalidixic acid, ampicillin, chloramphenicol at concentrations of 50 μ g/ml, 150 μ g/ml and 30 μ g/ml, respectively, supplemented with IPTG and X-gal. Plasmids with the mutated supF genes make E.coli cells resistant to nalidixic acid, while the cells carrying plasmids with the unmutated supF genes cannot grow in the presence of nalidixic acid. IPTG and X-gal further confirm the selection of the mutated *supF* gene by color of the colonies. A part of the bacteria was also plated on plates containing ampicillin and chloramphenicol to measure the fraction of the transformants.

Table 1 Classes of mutations induced in acetaldehyde-treated pMY189*.

Class of mutation	No. (%) of plasmids
Plasmids which have	
Single base substitution	24 (23.5)
2 base substitutions more than 3 bases apart	8 (7.8)
Tandem base substitution	
One tandem base substitution	53 (52.0)
Two tandem base substitutions	3 (2.9)
One tandem base substitution and one base substitution	7 (6.9)
One tandem base substitution and delesion	1 (1.0)
Frameshift	0 (0.0)
Delesion more than 2 base pairs	5 (4.9)
Insertion more than 2 base pairs	1 (1.0)
Independent plasmids sequenced	102 (100)

*Fourteen and 88 mutant plasmids were analyzed from 109 and 721 mutant clones of pMY189 obtained with treatment of 2M and 1M of acetaldehyde respectively.

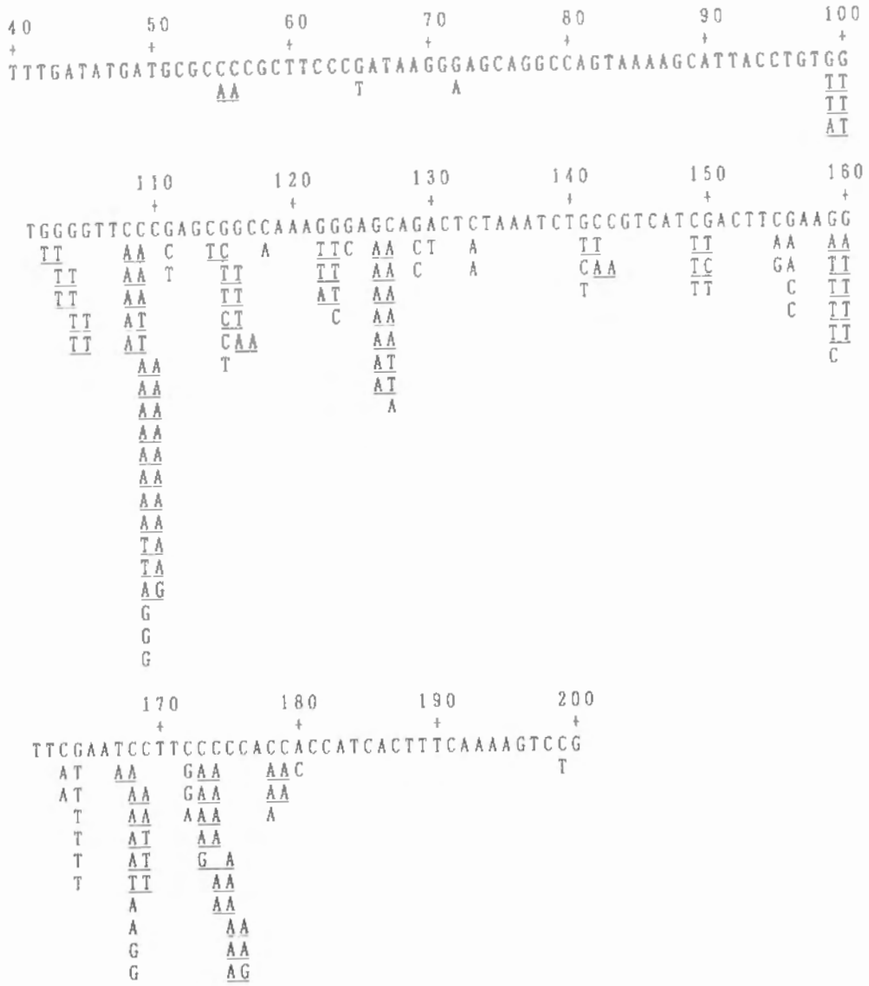

Fig. 2. Location of base substitution mutations found in the *supF* gene. Each letter represents a base substitution. Tandem base substitution mutations are underlined. Mutated plasmids were purified and the sequences of the *supF* gene were determined with the -21M13 primer and the Dye-Primer Cycle Sequencing reagent Kit using a 370A automatic DNA sequencer (Applied Biosystems).

followed by incubation for 3 days to allow repair, mutation fixation, and replication. The plasmids were extracted and transfected to the indicator *E.coli* strain to detect acetaldehyde-induced mutations and plasmid survival.

The acetaldehyde-treatment of pMY189 caused a dose-dependent reduction of the ampicillin-resistant bacterial colonies, and a dose-dependent increase in the mutation frequency of the *supF* gene (Fig. 1). Base sequence analysis of 102 mutant supF genes (Table 1) revealed that 63 % of mutated plasmids had tandem base substitutions, which has scarcely been reported to be induced by other mutagens except ultraviolet light(10) and reactive oxygens(11). Distribution of the base substitutions in the *supF* gene is shown in Fig. 2. The tandem base substitutions are present in various sites throughout the gene with a clean hot spot involving the base 109. The types of base substitutions are listed in Table 2. Eighty percent of the tandem base substitutions are located at CC (GG) sites, 13 % are located at 5'-GC-3' sites and 5 % are at 5'-CG-3' sites. The most predominant type of the mutations is CC → AA (GG → TT) (59.7%) transversions, which have rarely been reported with other mutagens previously, with an exception of cis-diamminedichloroplatinum(II) (12). In the base substitutions except the tandem base substitutions, 96% took place at G:C base pairs, G:C → T:A (46.8%) and G:C → C:G (38.3%) transversion mutations being the major types of the mutations.

As the treatment with acetaldehyde causes very specific base changes in

Table 2 Types of tandem and other base substitution mutations induced in acetaldehyde-treated pMY189

Type of mutation	No. (%) of base substitutions	Type of mutation		No. (%) of base substitutions
Tandem base substitutions	67 (100)	Other base substitutions		47 (100)
CC → AA (GG → TT)	40 (59.7)	C → T	(G → A)	5 (10.6)
CC → TT (GG → AA)	2 (3.0)	C → A	(G → T)	22 (46.8)
CC → TA (GG → TA)	2 (3.0)	C → G	(G → C)	18 (38.3)
CC → AT (GG → AT)	6 (9.0)	T → C	(A → G)	0
CC → AG (GG → CT)	3 (4.5)	T → A	(A → T)	1 (2.1)
GC → AA (GC → TT)	7 (10.4)	T → G	(A → C)	1 (2.1)
GC → AT	2 (3.0)			
CG → TC (CG → GA)	2 (3.0)			
CG → TT (CG → AA)	1 (1.5)			
TC → AA (GA → TT)	1 (1.5)			
CCC → GCA (GGG →TGC)	1 (1.5)			

DNA, the base sequence change may be used as a probe to identify acetaldehyde as the causal agent in human tumors. Seventy percent of human tumors have been shown to contain mutations in the p53 gene(13), and the specificity in types of mutations in this gene could be related to the causal environmental agents, such as sunlight UV to CC→TT tandem double mutations in skin tumors(10,14-16), and afratoxin B1 to G→T transversions in liver tumors(17,18). Only one CC → AA(19) tandem base substitution was found in the analyses of 541 base substitutions in the p53 gene in human cancer in various organs, by our survey of 41 literatures. Among other types of changes, one CC→TT(20) and one CG→AA(21) tandem base substitutions were the only ones similar to what found in this report. This strongly suggests that the tandem base substitutions are very rare, and if detected, may represent the specific cause responsible for such changes. Two of these tandem base substitutions were found in lung(19,20) and the remaining one was in esophagus(21), and could be related to the smoking and the alcoholic baverage, although no details concerning the smoking or drinking history of the subjects concerned were given. Another possible agent responsible for the tandem base substitution is reactive oxygens(11), with frequent CC→TT changes, but never with CC→AA changes, and the specificity was not so high as with acetaldehyde. The CC→AA tandem base substitutions, coupled with the epidemiological survey of the smokers and drinkers may reveal the burden due to acetaldehyde.

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Chapter 6

Conclusion of This Thesis

Measurement of DNA strand breaks by FADU method

We tried to apply FADU method as a short-term test to detect DNA-damaging environmental agents, using an SV40-transformed human fibroblast cells. We can quantify the number of DNA strand breaks using the dose-response curve of γ-rays as a calibration curve of DNA strand breaks (Fig. 1 of Chapter 2). DNA strand breaks are induced by three mechanisms. The first mechanism is the direct breakage of phospodiester linkages of DNA induced by such as γ-rays, reactive oxygens and some anticancer drugs like bleomycin. The second, DNA breakage is induced by DNA repair endonucleases. The third mechanism is the apoptotic cell death. We tried to detect DNA strand breaks caused by DNA repair endonucleases as well as the direct breakage and apoptotic DNA fragmentation, for many DNA damaging agent does not induce strand breaks directly. We used DNA polymerase inhibitor araC and hydroxyurea to prevent strand rejoining for sensitization of this assay (Fig. 1). We can detect DNA strand breaks induced by physical and chemical carcinogens such as γ-rays, UV light, MNNG, 4NQO, B(a)P efficiently by using araC and hydroxyurea.

We applied this method to evaluate the DNA damage in human cells

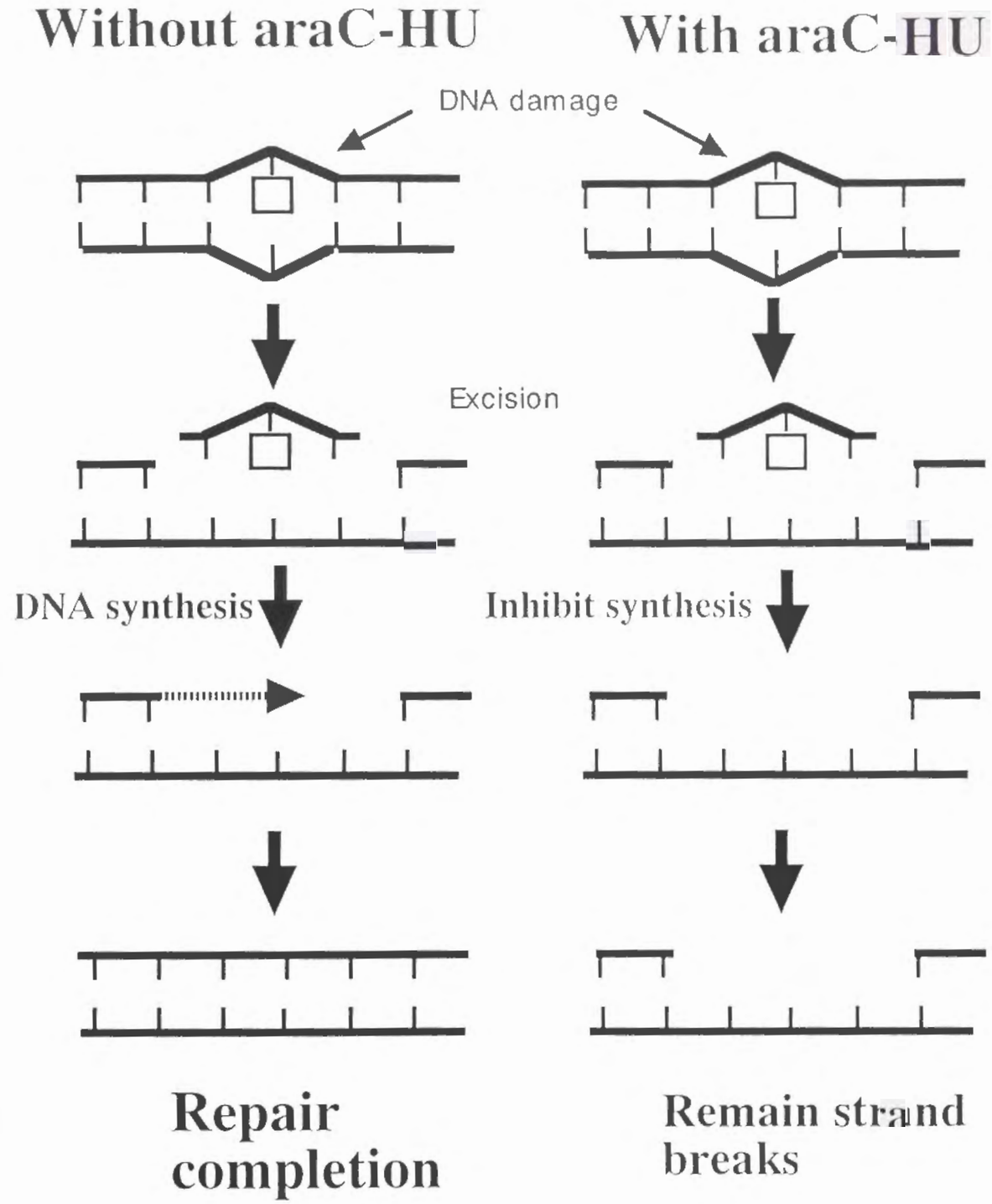

Fig. 1 Use of DNA polymerase inhibitor araC and hydroxyurea for sensitization of the FADU assay.

induced by contaminants in tap water. We compared DNA damage induced by the tap water of Kyoto City and Osaka City. The tap water of Osaka City showed much higher potential to induce DNA damage in human cells than tap water of Kyoto City. This may reflect the water quality of the source of municipal water supply. This result is not contradictory to our previous findings, using the *Bacillus subtilis* rec-assay, that DNA damaging potential of contaminants in the Yodo River increased as the river goes down. Rat liver S9 fraction and glutathione and boiling of the tap water reduce the DNA damaging potential. These result suggest that the risk of tap water to human beings is reduced by boiling of water for cooking or making tea, and the risk is further reduced by enzymes and glutathione in the human body.

Although the FADU method is useful to evaluate DNA damage in human cells, there are some limitations and dilemmas when we use this method for risk assessment. First, we can not detect DNA strand breaks induced by DNA crosslinking agents such as mitomycin C or cis-diamminedichloro-platinum(II), because DNA interstrand crosslinks inhibit alkaline unwinding of double-strand DNA. Second, we can not detect DNA damages if these damages are not repaired in human cells. So we can not extend the results of this assay directly, to evaluation of mutagenicity or carcinogenicity to human beings. For example, very strong mutagens IQ, MeIQ and MeIQx does not induce DNA strand breaks in human cells (data not shown). It is

necessary to combine the FADU assay with other assays of gene mutation, clastogenicity and promotion activity in human cells to evaluate the actural cancer risk to human beings.

Mutation spectrum of the 2-chloroacetaldehyde and acetaldehyde.

We analyze the mutation spectrum induced by two aldehydes, 2-chloroacetaldehyde and acetaldehyde. Both aldehydes are thought to be related to human cancers. The 2-chloroacetaldehyde is the structurally similar compound of acetaldehyde, only one hydrogen atom in the methyl group of acetaldehyde is took place to the chlorine atom (Fig. 2). 2-chloroacetaldehyde mainly induce G:C to A:T transition at G:C pairs in 5'-AAGG-3' or 5'-CCTT-3' sequences. Acetaldehyde shows quite different pattern of mutations from 2-chloroacetaldehyde. Sixty-three percent of mutations acetaldehyde induced are the tandem mutations, while, 2-chloroacetaldehyde-induced tandem mutation is only 0.9 percent (Fig. 3). Although we do not know the structure of acetaldehyde-induced DNA damages which read to tandem mutations, they are probably the dimers of guanine or cytocine bases. It is well known that aldehydes form crosslinks between amino groups of DNA. The possible structures of the acetaldehyde-induced DNA lesions are indicated in Fig. 4. A carbonyl group in acetaldehyde can bind to an amino group of guanine or cytocine and make a

crosslink between the adjacent two bases. It is important to elucidate the mechanisms of induction of tandem mutations in order to estimate the cancer risk of exposure of acetaldehyde to human beings.

In conclusion, 2-chloroacetaldehyde and acetaldehyde showes very specific mutation spectrum, and these spectrum may be used as a probe to identify these aldehydes as the causal agent in human tumors.

CH_2Cl-CHO

2-chloroacetaldehyde

CH_3-CHO

Acetaldehyde

Fig.2 The structure of the 2-chloroacetoaldehyde and the acetaldehyde.

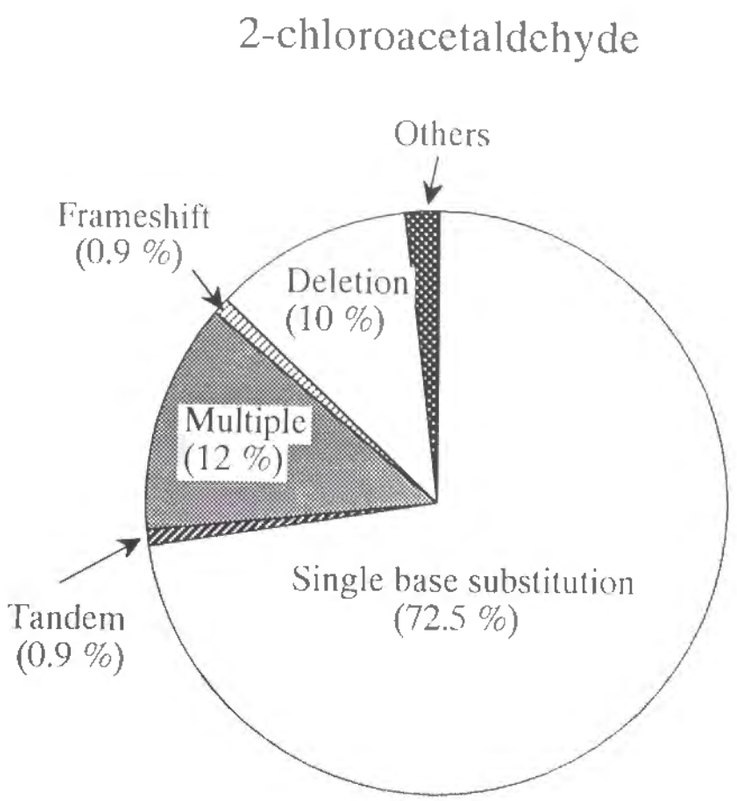

Fig.3 Types of the mutations induced by the 2-chloroacetaldehyde and the acetaldehyde.

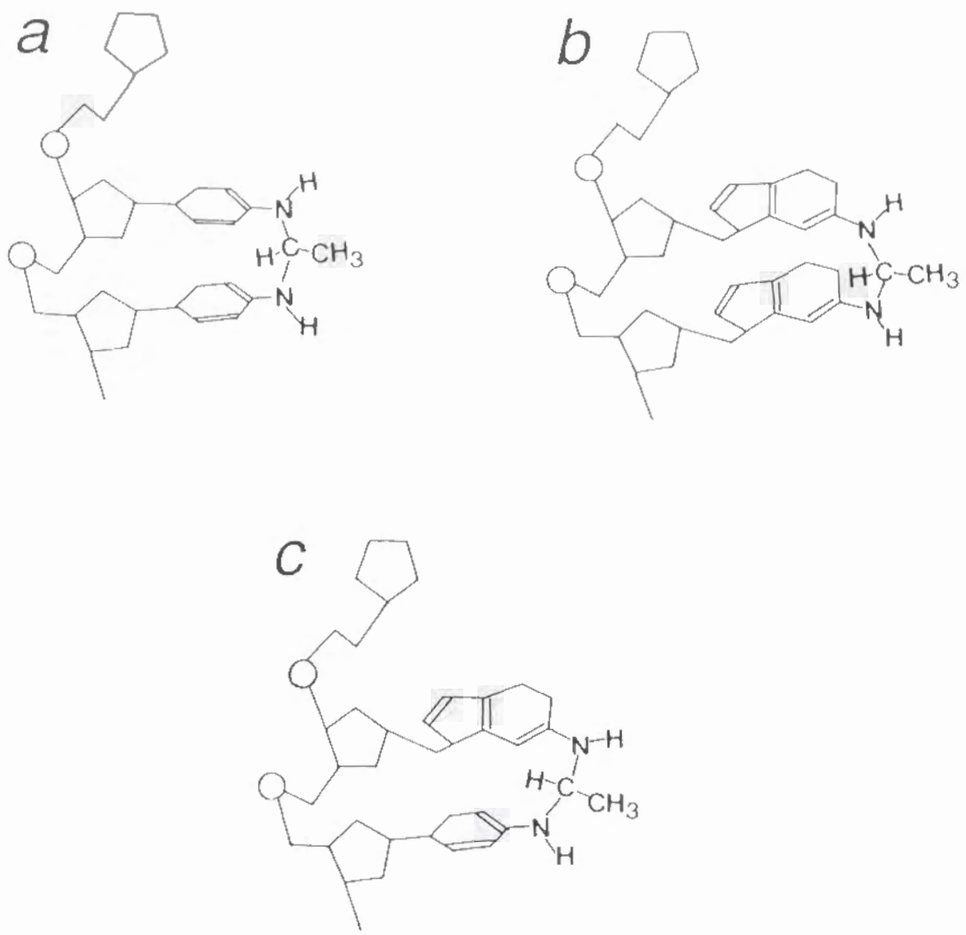

Fig.4 The possible structure of the acetaldehyde-induced DNA lesions. a, dimmer of the cytosine. b, dimmer of the guanine. C, guanine-cytosine heterodimmer.

List of Publications

Chapter 2

松田知成、八木孝司、武部啓、松井三郎　環境工学論文集・第３０巻 p227-234, 1993

Chapter 3

To be submitted to Mutation Research

Chapter 4

Submitted to Cancer Research

Chapter 5

Submitted to Nature

Other Publications

The following papers, which are not included in this thesis, have been published by author et al.

S. Matsui, N. Semba, T. Matsuda and H. Yamada, Wat. Sci. Tech. vol. 25, p301-308, 1992

松井三郎、川口英之、仁井敦、松田知成　環境技術 vol. 21 p776-787, 1992